JULY 10, 1941

THE JOURNAL OF NUTRITION

VOLUME 22 NUMBER 1

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Official Organ of the American Institute of Nutrition
PUBLISHED MONTHLY BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA, PA.

Price, $5.00 per volume, Domestic; $5.50 per volume, Foreign

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SEVERE CALCIUM DEFICIENCY IN GROWING RATS

III. SERUM CALCIUM OF INDIVIDUAL ANIMALS DURING DEVELOPMENT OF CALCIUM DEFICIENCY [1]

DAVID M. GREENBERG AND WILBUR DOANE MILLER
Division of Biochemistry, University of California Medical School, Berkeley

ONE FIGURE

(Received for publication January 31, 1941)

The results of a study of the symptoms, the pathology, and the changes in chemical composition of rats suffering from uncomplicated, severe, calcium deficiency have recently been published by Boelter and Greenberg ('41). It was demonstrated, in the course of this study, that a marked reduction in the serum calcium concentration occurred. To demonstrate this fact it was necessary to sacrifice groups of animals at varying intervals of time, and analyse their pooled blood.

It seems highly desirable to follow the changes in serum calcium in the same animal during the whole course of the calcium deficiency. The successful application by Lindner and Kirk ('37) of the cerate-Mohr's salt titration procedure to the quantitative estimation of calcium in a single drop of blood serum made such an undertaking feasible, and led us to undertake the investigation.

The results obtained show that although there are considerable daily fluctuations, development of a severe calcium deficiency leads to a progressive decline in the serum calcium concentration. The serum protein concentration remains unchanged. It is interesting that even though the serum calcium level was greatly lowered, at no time did the rats on the low calcium regimen show any indications of tetany.

[1] Aided by a grant from the Christine Breon Fund for Medical Research. Technical assistance was furnished by the personnel of W. P. A., official project no. 65-1-08-62.

THE JOURNAL OF NUTRITION, VOL. 22, NO. 1
JULY, 1941
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EXPERIMENTAL

The investigation was carried out upon eight rats of the Long-Evans strain reared in our stock colony. After being weaned at 28 days of age, the rats were kept on the stock colony diet for a week or more until they weighed about 100 gm. each. They were then placed on the calcium-low diet. This procedure was followed in order to have heavier animals that would stand the successive bleeding better than those that are ordinarily used in the calcium-deficient studies. The starting range in weight was from 94 to 125 gm., and the mean weight was 107 ± 12 gm.[2] When they were sacrificed after 82 to 134 days on the low-calcium regimen, the mean weight was 161 ± 40 gm. At this time all of the animals had dropped below their maximum weight which averaged 199 ± 40 gm. The maximum weight was attained at anywhere from 32 to 68 days after the animals were placed on the deficient diet.

The experimental diet contained about 10 mg. of calcium per 100 gm. of food. Its composition has been reported in a previous publication (Kleiber, Boelter and Greenberg, '40).

The apparatus and technique of Kirk ('33) were used for the drop analysis. Blood, obtained at intervals of 2 to 6 days by snipping the tail, was collected in micro-centrifuge tubes and, after centrifugation, measured samples (about 0.05 ml.) of the separated serum were pipetted into 3 ml. capacity silica ware crucibles, and the pipettes, calibrated for capacity, were rinsed out with an equal volume of redistilled water. The serum was dried in an electric oven maintained at about 80°C. as a preliminary to ashing. The crucibles were then transferred to a micro-muffle furnace and the serum ashed by adjusting the temperature to 400-500°C. After 45 minutes of heating, the temperature was raised to 500-550°C. for about 10 minutes. The ash was dissolved in about 1 drop of 0.1 N hydrochloric acid, and the calcium precipitated as the oxalate in the silica crucible. The precipitate was washed

[2] The measure of variability used in this paper is ± standard deviation.

by means of a sintered glass, micro external filter. After washing, the calcium oxalate was dissolved in a drop of 6 N sulfuric acid, and the oxalic acid oxidized by addition of a measured excess of standard ceric sulfate. The remaining cerate ion was titrated with ferrous ammonium sulfate from a capillary burette in the presence of o-phenanthroline as indicator. The analysis was carried out by following the procedure of Lindner and Kirk ('37) except for a few modifications which are given below.

With the exception of the ceric sulfate standard solution, which remained stable indefinitely, it was found desirable to prepare the solutions for use from carefully protected stock solutions and recrystallized salts about twice a month. The asbestos suspension for filtering the calcium oxalate was left to soak in the acid-ceric sulfate solution, and only a small quantity was prepared for immediate use as described by Lindner and Kirk. The o-phenanthroline indicator solution was added directly to the solution of the Mohr's salt (about 15 drops to 30 ml.) before the titration rather than being introduced into the solution containing the sample following the addition of the ceric sulfate. This procedure eliminates a blank for the titer of the indicator, since the o-phenanthroline is kept in the reduced stable form by the Mohr's salt.

To prevent gross errors, the analysis on such minute samples must be performed with great care to avoid contamination. In the present work all analyses were performed in duplicate, and the average difference between two duplicate analyses was about 4%. Occasional results were obviously in great error and these were discarded.

RESULTS

The curve of the change in blood serum calcium concentration with time on the low calcium diet is shown in figure 1. The analytical values for each experimental animal are indicated by a distinctive symbol. The solid line represents the average curve for all eight rats. It is to be observed that the calcium level commences to drop almost at once after

the animals are placed on the experimental regimen, although the concentration does not fall below the normal range until after about 2 weeks on the diet. After 30 days, the average serum calcium concentration has fallen to 7.4 mg. per 100 ml., and at 68 days it is 6.1. The rate of fall is greatest for the first 40 days, after which the serum calcium concentration tends to become stabilized. Rats killed at 82 and 133 days

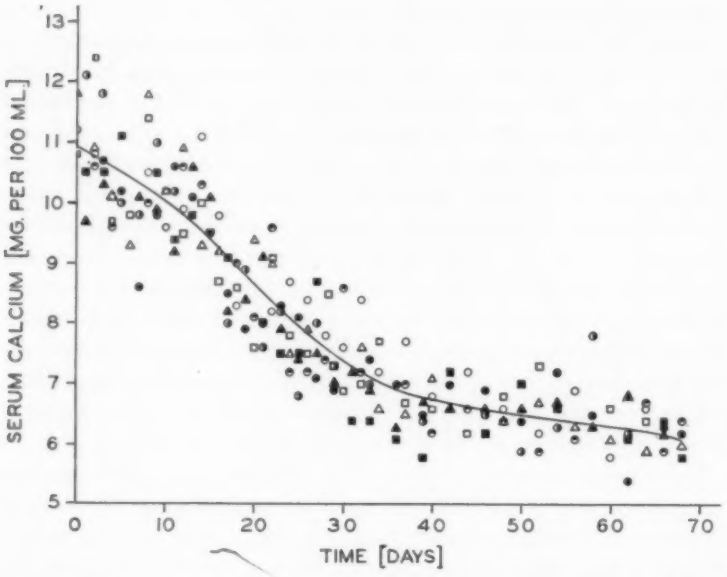

Fig. 1 The change of the serum calcium concentration with time in individual rats on a diet very low in calcium.

showed essentially the same serum calcium concentration as is given by the curve at 68 days.

The serum protein level is not changed by the calcium deprivation. Analysis of the serum protein on a series of nine calcium deficient rats by the method of Greenberg and Mirolubova ('36) gave an average value of 6.65 ± 0.85 gm. protein per 100 ml. of serum.

After 9 weeks on the diet, some of the animals were subjected to a galvanic shock, producing paralysis and symptoms of hemorrhage (Boelter and Greenberg, '41), but this caused no noticeable change in the serum calcium concentration. The animals given the galvanic shock lost a great deal of weight, which is one of the reasons the mean final weight is lower than the mean maximum weight of the animals.

Analyses of body calcium and phosphorus were carried out on five of the animals after they were sacrificed. This yielded values of $0.426 \pm 0.06\%$ calcium and $0.383 \pm 0.03\%$ phosphorus as against the normal values of 1.0 and 0.75%, respectively. The comparison shows that a great reduction in body calcium and phosphorus occurs on the low calcium diet. Depletion of the calcium stores of the animal apparently is necessary to produce a lowering of the serum calcium concentration in rats with an intact thyroid-parathyroid apparatus.

SUMMARY

1. The change in serum calcium concentration in rats on a severely calcium deficient diet has been followed in the same animal during the whole course of the deficiency by means of analysis upon a single drop of blood serum.

2. Development of severe calcium deficiency resulted in a progressive decline in the serum calcium until the concentration of calcium fell to about 6 mg. per 100 ml. of blood serum. The serum protein concentration was not altered by calcium deprivation.

We are greatly indebted to Dr. M. D. D. Boelter for carrying out the analysis of the serum protein and the body calcium and phosphorus.

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FURTHER OBSERVATIONS OF RIBOFLAVIN DEFICIENCY IN THE DOG [1]

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TWO FIGURES

(Received for publication December 30, 1940)

Street and Cowgill ('39) reported certain observations of riboflavin deficiency in the dog, but numerous other phenomena remained to be described. These are presented in the present paper. Among other effects, degenerative changes in the nervous system have been observed.

EXPERIMENTAL PROCEDURE

The animals used for this study were fifteen adult mongrel dogs, ranging in weight from 4.85 to 8.6 kg. at the beginning. All were given a vermifuge before the experiment began.

Diet employed. The basal diet used has already been published (Street and Cowgill, '39). Certain other pertinent details to be mentioned are as follows: The control animals received as a supplement to the basal diet 25 μg. of riboflavin [3] per kilogram of body weight daily. The provision of the water-soluble vitamins needed by the dog other than riboflavin was effected by the administration of a tikitiki prepared by ourselves. Growth tests with rats indicated that

[1] The expenses of this investigation were defrayed by grants from the Rockefeller Foundation and the Research Fund of the Yale University School of Medicine.

[2] Vitamin Research Fellow, 1936–1938.

[3] Obtained from the Borden Company, Bainbridge, New York, as crystalline lactoflavin, PX grade (highest purity).

this product contained only about 3 to 4 μg. of riboflavin per cubic centimeter; at the level of 0.5 cc. per rat daily, this tikitiki, given as the sole source of water-soluble vitamins, permitted only poor growth, i.e., an average of 0.77 gm. per day during the first 8 weeks, for fifteen rats. Most of these animals developed moderate to extensive loss of hair, characteristic of flavin deficiency, as well as other symptoms of an insufficient supply of flavin. Six rats given daily 0.5 cc. of the tikitiki plus 40 μg. of crystalline riboflavin as their source of the water-soluble vitamins grew at the reasonably satisfactory rate of 2.66 gm. per day during a period of 8 weeks. These animals appeared completely normal in every respect.

The tikitiki, when assayed for vitamin B_1 by the pigeon method (Block, Cowgill, and Klotz, '32) was found to contain 3.3 pigeon units per cubic centimeters, or approximately 8 international units per cubic centimeter. It was fed to the dogs in the amount of 0.5 cc. per kilogram of body weight daily (4 I.U./kg.) which is about three times the amount of vitamin B_1 estimated to be required by dogs of this size (Cowgill, '34).

Grouping of animals. The dogs used in this study may be classified into three groups, according to the dietary regimens upon which they subsisted.

Group 1. Five dogs were given only the basal diet until the development of sudden collapse characteristic of dogs maintained on this ration (Street and Cowgill, '39). Four of the animals, after collapse, were injected with a solution of riboflavin, the dose used being approximately 0.75 mg. per kilogram of body weight. Three of the dogs recovered and were then continued on the basal diet without further riboflavin until they collapsed for the second time. The second collapse was fatal in each case, although two of the dogs were injected with riboflavin late in the course of the attack.

Group 2. Four dogs were given small daily doses of flavin (4 to 8 μg. per kilogram of body weight), amounts which were thought to be sufficient to support life but not enough to main-

tain health. Thus, it was hoped, a condition of chronic deficiency would be produced. The dogs of groups 1 and 2 were offered daily an amount of the basal mixture furnishing approximately 90 calories per kilogram of body weight.

Group 3. Six dogs served as inanition controls for certain of the dogs of the two preceding groups. Each control was paired with an experimental animal of similar body weight and was given daily that amount of the basal mixture eaten by the experimental partner the preceding day. In addition, the control dogs received daily 25 μg. of riboflavin per kilogram of body weight. Thus, the only difference in the treatment of the experimental and control dogs was the amount of riboflavin given as a supplement to the basal diet.

Clinical observations. The animals were weighed weekly and were examined frequently for any abnormalities such as loss of hair, sore mouth, dermatitis, cataract, or corneal opacity. Each dog was taught to run on a turn table, perform on an obstacle board, and stand on its hind legs; records were kept throughout the experiment of any changes in the ability to perform these tasks. The dogs that developed acute riboflavin deficiency were autopsied immediately after death. The animals of group 2, as well as the control dogs, were chloroformed and autopsied at the conclusion of the experiment.

Histologic technique. The brain and spinal cord and the sciatic, brachial, and usually the vagus nerves were fixed immediately after removal in 95% alcohol, 10% neutral formalin and Müller's solution (without formaldehyde). The nervous tissues were stained by the Marchi, Sudan III, Kultschitzky and Nissl techniques. Sections of the liver and of some of the other viscera were stained with hematoxylin and eosin.

RESULTS

General behavior of the animals. We have previously described ('39) the behavior of dogs maintained on the basal diet alone. All such animals collapsed in a characteristic manner, usually in from 100 to 150 days after beginning the

experiment. Animals will recover from this state of collapse only if given riboflavin before the attack has progressed too far. Aside from the collapse, the chief effects of the diet observed in the animals of group 1 were lessened activity and a moderate decrease in appetite. The dogs of group 2 also exhibited decreased appetite and activity after a considerable period.

In contrast to the symptoms seen in rats on such a diet, loss of hair was not observed in these animals except that the hair wore thin on the chests of two dogs after they became inactive and spent most of the time lying down. No soreness of the mouth was seen except in two dogs. Experimental dog 7 and control dog 8 both developed a unilateral necrotic bleeding area on the mucosa of the cheek opposite the last upper molars. It was the opinion of Dr. Bert G. Anderson [4] that the ulcers were an effect of the irritation of the cheek by the thick deposits of calculus on these teeth. Removal of the calculus from the teeth of one of the dogs caused temporary improvement.

The skin of the dogs in group 1 (acute deficiency) remained healthy. However, two of the animals in the chronic deficiency group (dogs 9 and 13) exhibited a persistent scaling of the skin of the abdomen and medial surface of the hind legs, similar to that described by Sebrell and Onstott ('38). Large, thin, loose scales of skin were constantly present. This was accompanied by a very slight erythema.

With a few exceptions, the animals lost considerable weight; these weight changes are shown in table 1. It will be seen that in the acute deficiency group the weight losses were

TABLE 1

Weight losses in riboflavin deficiency

	GROUP 1 ACUTE DEFICIENCY					GROUP 2 CHRONIC DEFICIENCY				GROUP 3 CONTROL					
DOG NO.	1	3	53	55	57	7	9	13	51	2	6	8	10	12	52
Initial weight (kg.)	8.6	7.3	6.0	7.9	7.4	7.2	6.0	4.9	6.0	8.6	7.5	7.1	6.6	5.8	6.6
Final weight (kg.)	6.0	9.0	3.6	5.3	4.8	5.2	5.9	3.7	4.9	7.3	5.4	4.1	7.2	5.0	5.0
Weight loss (%)	30	..	40	33	41	28	2	25	18	15	28	42	..	14	24

[4] Associate Professor of Surgery, Yale University School of Medicine.

from 30 to 41%, except for one animal which gained weight. In the chronic deficiency group three of the dogs lost 19 to 28%. The weight of dog 9 for the major part of the experiment remained close to the initial weight of 6.0 kg. There was, however, a period during which the weight dropped slowly to 4.75 kg. The weight at the end of the experiment was 5.85 kg. The six control dogs lost from 13 to 42% of their weight, with the exception that dog 10 gained 0.6 kg. This gain in weight on a limited food intake was in part explained by the fact that this particular animal was very inactive.

Condition of the eyes. Five dogs showed an abnormal condition of the eyes; three of these were in group 1 (acute deficiency), two in group 2 (chronic deficiency). No abnormalities were seen in the eyes of the control dogs.

Group 1. On the one hundred and thirty-sixth day of the experiment it was observed that the eyes of dog 3 were half-closed, the nictitating membranes pulled forward until they covered about a third of the eye, and the eye balls rotated dorsally. On the one hundred and thirty-eighth day the eyes were no longer rotated this way, but the eyelids were still half-closed. On the one hundred and fortieth day the condition was the same. On the one hundred and fiftieth day and for the remainder of the experiment the eyes looked normal. It is to be noted that the dog appeared on the verge of collapse on the one hundred and thirty-sixth day, remained inactive for 4 days, then spontaneously recovered much of its vigor by the one hundred and forty-fifth day.

In the case of dog 55, it was observed that the left eye was half-closed on the one hundred and fifth day. This condition disappeared after 2 days. On the one hundred and twenty-first day it was noticed that the right eye was kept partially closed. This also returned to normal within a few days. On the one hundred and eightieth day it was found that there were opacities of the cornea in both eyes, that in the left eye measuring about 3×4 mm., the measurements of that in the right eye being somewhat smaller.

In dog 57 on the two hundred and sixth day of the experiment, the day preceding death, it was observed that the right cornea had a dry appearance and was indented in many places, giving a pebbled effect. There was no perceptible opacity and there did not appear to be any breakdown of the surface. The left eye appeared perfectly normal.

Group 2. Opacities of both corneas were observed in dog 7 on the three hundred and ninty-seventh day, measuring about 3 mm. by 1 mm. in the left eye and a little smaller in the right. By the four hundred and eleventh day the opaque spot in the right cornea had increased somewhat in size. The condition was described by Dr. Edward N. DeWitt [5] on the four hundred and fifty-fourth day as follows: "There are opacities in the stroma layer of the cornea of each eye, and scattered for 2 or 3 mm. beyond the main mass are small dot-like opacities in each eye which remind one of deep punctate keratitis. The rest of each eye is perfectly normal." In the left eye the opacity measured 5 mm. horizontally by 2.5 mm. vertically, and in the right eye 4 mm. horizontally by 2 mm. vertically. It was found on the four hundred and ninety-first day that the left eye was watering, and by the five hundred and thirty-ninth day both eyes were watering, forming brown stains on the animal's white coat running down from the anterior corner of each eye.

Opacities were discovered in the eyes of dog 13 on the two hundred and sixty-fifth day of the experiment. They appeared as sharply circumscribed light spots in the center of each cornea, measuring about 3 to 4 mm. in the transverse direction by about 1.5 mm. vertically. On the two hundred and ninety-third day there was an area of opacity around the original spot in the left eye: the outer area was not so intensely opaque, but paler. Both eyes were watering considerably.

Doctor De Witt described the appearance of the eyes on the three hundred and sixty-fourth day in the following words:

[5] Assistant Clinical Professor of Ophthalmology, Yale University School of Medicine.

"Left eye: Externally the eye is normal. There is a normal reaction to light. There seems to be a slight epipheria. *Cornea:* external epithelial layer normal and clear. In the stroma tissue there is an opacity measuring horizontally 5 mm., vertically 2.5 mm. Its general shape is oval, and it has a glistening, shining, water-like sheen. Toward the inner canthus the opacity is somewhat denser, and has a yellowish glistening hue, which resembles early calcareous degeneration. *Anterior chamber:* normal in depth and clear. *Lens:* normal, perfectly clear, no opacities. Vitreous, choroid, retina, nerve-head, vessels normal. *Right eye:* The external appendages are the same as those of the left. There is an opacity in the stromal tissue of the cornea resembling the one in the left eye, but it is much smaller, measuring 3 mm. horizontally and 2 mm. vertically."

From the four hundred and first day till the end of the experiment on the four hundred and fifty-seventh day the eyes were watering noticeably, and on one occasion were found to be stuck shut in the morning.

The effect of varying the amount of riboflavin given to the animals of group 2. Dog 7 received daily 55 μg. (8 μg./kg.) from the thirty-fifth to four hundred and thirty-fourth day, and 28 μg. (4 μg./kg.) from the four hundred and thirty-fifth to five hundred and fortieth day. Dog 9 received daily 48 μg (8 μg./kg.) from the twenty-fourth to two hundred and sixty-seventh day, 25 μg. (4 μg./kg.) from the two hundred sixty-eighth to three hundred and twenty-third day, 48 μg. (8 μg./kg.) from the three hundred and twenty-fourth to four hundred seventy-third day, and 35 μg. (6 μg./kg.) from the four hundred and seventy-fourth to four hundred and ninety-third day.

In addition, the following single injections were given to dog 9: 2.25 mg. on the three hundred and thirty-second day, 1 mg. on the three hundred and seventy-first day, 1 mg. on the four hundred and second day.

Dog 13 was fed the basal diet only from the first to one hundred and forty-ninth day; then it received 20 μg. (4 μg./kg.) of riboflavin from the one hundred and fiftieth to three hundred and sixty-first day; and the basal diet only from the three hundred and sixty-second to four hundred and fifty-seventh day.

Dog 51 was given 48 μg. (8 μg./kg.) of riboflavin from the eighth to one hundred and ninety-second day, and 25 μg. (4 μg./kg.) from the one hundred and ninety-third to two hundred and eighth day.

Since dog 9, after 267 days, had maintained its weight at the initial level of 6.0 kg. while receiving 8 μg. of riboflavin per kilogram, it was decided to cut the flavin dosage in half. The animal at once began to lose weight (fig. 1) and soon became less active. The dose of flavin was increased to 8 μg./kg.

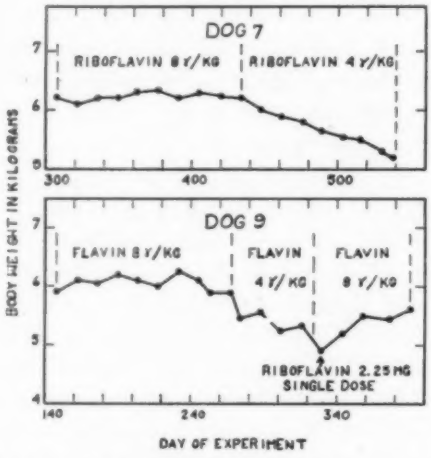

Fig. 1 Graph showing the loss in weight of two dogs when the daily dose of riboflavin was reduced to 4 micrograms per kilogram of body weight after a long period of maintenance with 8 micrograms of riboflavin per kilogram.

again on the three hundred and twenty-fourth day, but despite this, the dog developed the characteristic collapse of acute riboflavin deficiency on the three hundred and thirty-second day. It was restored by the injection of 2.25 mg. of riboflavin.

The riboflavin supplementation for dog 7 was reduced from 8 μg./kg. to 4 μg./kg. on the four hundred and thirty-fifth day, to see whether it would be possible to hasten the development of certain symptoms of insufficient flavin which had appeared

in an early form. Up to this time the weight had been constant at 6.1 to 6.3 kg. for the preceding 125 days. The body weight at once began to decline, gradually but steadily, decreasing to 5.2 kg. by the five hundred and fortieth day. These weight changes are shown graphically in figure 1.

Dog 13 behaved in a somewhat anomalous manner; although given no flavin for the first 149 days the characteristic collapse did not appear; the animal did lose a little weight. Supplementation with 4 μg. riboflavin per kilogram for the next 212 days prevented any appreciable further loss of weight. This dog was occasionally observed to eat its feces. Presumably this explains her unusually low requirement for riboflavin, since it has been our experience that coprophagy protects dogs from deficiency of the water-soluble vitamins.

Neurological dysfunctions

Numerous observations were made of the ability of the animals to perform on an obstacle board and a turn table as well as to balance themselves on their hind legs. The type of information yielded by these tests and the interpretation placed on them by a clinical neurologist are illustrated in the remarks of Dr. James C. Fox, Jr.,[6] concerning animals 7, 9 and 13 in the chronic deficiency group and control dog no. 8 presented in table 2. In the acute deficiency dogs of group 1 some earlier signs of neurological dysfunction were noted in some but not all of the animals usually after about the ninetieth day of the dietary regime (dogs 3, 53 and 55). In contrast to these findings may be cited the fact that the group of control dogs remained in apparently perfect health throughout the experiment (see no. 8 in table 2); they were lively, had good appetites, and showed no neurological abnormalities although observations were made as late as the five hundred and thirty-fifth day of the experiment.

Histologic studies

Nervous system. A summary of the findings pertaining to the pathology of the nervous system is presented in table 3.

[6] Clinical Professor of Neurology, Yale University School of Medicine.

TABLE 2

Illustrative reports of neurological examination of animals

DOG NO.	REMARKS
	Chronic riboflavin deficiency
7	4/7/38—426th day: "The dog gives the impression of being a little asthenic without true muscular weakness or ataxia in any of the tests. Treads and stands on hind legs well; reflexes active."
	6/25/38—505th day: "The dog has definitely changed since last examined on 4/7/38. The ordinary gait appears normal in contrast to that exhibited on the turn table which is abnormal. Throws hind legs on obstacle board; slides on turn table; refuses to stand on hind legs. Wavers when supported by fore paws and is a bit insecure in jumping. Fore paws apparently not affected. Shaking reaction is normal. Deep reflexes all diminished markedly, the knee jerks just barely obtained. *Impression:* Weakness in hind legs. The loss of the deep reflexes suggests lower neuron involvement. Whether there is any ataxia of higher origin cannot be determined; probably not."
	7/25/38—535th day: "Performance in general is similar to that described on 6/25/38. He does stand on his hind legs, insecurely, today. The deep reflexes cannot be obtained anywhere, even in the proximal portions of the limbs. When supported by the hind legs, the fore paws do not seem to have full strength. Although the unsteadiness was not increased significantly on the turn table, obstacle board, or when standing on the hind legs, he is definitely ataxic on shaking. He does not object to pin prick or hair pulling anywhere."
9	4/7/38—411th day: "The dog is rather pepless, sluggish on obstacle board, keeps a rather broad base, is able to stand on its hind legs, treads slowly, and is not thrown off balance easily. Deep reflexes are normal."
	6/25/38—490th day: "This dog has changed quite markedly since last examined on 4/7/38. It is generally weak, sluggish, short of breath on exertion; soon sits down and then rests on the floor. Weakness in the hind legs increases the longer it is upright, the 'compass gait' becoming more striking. It is unable to hurdle, and treads very clumsily. Deep reflexes are practically absent except for a feeble jerk in the proximal flexors. Shaking: not particularly ataxic but, in fact, quite stable in view of weakness of limbs. *Impression:* Lower motor neuron weakness, in both front and hind legs, and greater in the latter; generalized asthenia, and lowered cardiac reserve. It is very doubtful whether it has any ataxia of higher origin."
13	4/7/38—336th day: "This dog is in better condition neurologically than its two experimental colleagues, nos. 7 and 9. The impression is gained that it shows minimal lag and clumsiness of the hind legs on the obstacle board. This may be due to beginning weakness. Its behavior in other respects is normal, especially in the ability to stand on its hind legs unsupported. The knee jerks are still very lively. *Impression:* Beginning weakness of hind legs?"
	7/25/38—445th day: "Response the same as described in above note; possibly the right rear leg gives way a little more than the left after fatigue on the turn table or from walking on hind legs. Deep reflexes all lively."

Control—Companion of dog no. 7 paired-fed with basal diet but received in addition daily 25μg./kg. of riboflavin.

8 4/7/38—426th day: "Decidedly more alert and active than experimental partner; can tread faster and jumps when standing on hind legs. Is faster over obstacle board and slips a little less."
505th day: "No additional comments."
535th day: "Response is normal; reflexes active."

TABLE 3

Myelin degeneration in the different groups of animals as revealed by various stains compared with the length of period of subsistence on the deficient diet

DOG NO.	DAYS ON DIET	MARCHI		KULTSCHITZKY		SUDAN III		NISSL		PAIRED WITH EXPERIMENTAL DOG NO.
		Nerve	Cord	Nerve	Cord	Nerve	Cord	Cord	Brain	
					Acute deficiency group					
53	110	—	—	—	—	—	—	—	—	
1	152	+	—	—	—			—	—	
55	182	3+	+	—	—	—	—			
57	207	4+	3+	3+	+					
					Chronic deficiency group					
51	208	±	—	—	—	—	—			
13	457	3+	2+	2+	+			2+	—	
9	493	4+	4+	3+	3+			2+	—	
7	540	3+	3+	3+	3+			2+	—	
					Control group					
2	158	+	—	—	—			—	—	1
6	131	—	—	—	—			—	—	
8	547	+	—	—	—			—	—	7
10	512	+	±	—	—			—	—	9
12	542	±	±	—	—			—	—	13
52	211	+	—	—	—	—	—			51

Grading system of lesions: — none; ± minimal or questionable; + mild or slight; 2+ moderate; 3+ extensive; 4+ very extensive or extreme.

As indicated in the earlier work on the pathologic changes in the nervous system in avitaminosis (Zimmerman and Burack, '34; Zimmerman, Cowgill and Fox, '37), the essential lesion is a demyelination of the peripheral nerves and the posterior columns of the spinal cord (fig. 2A and 2B). In the chronic deficiency state gliosis replaces the injured posterior columns. In each instance where cord changes are present the peripheral nerves are also affected, but the reverse is not always true. From this and the fact that the

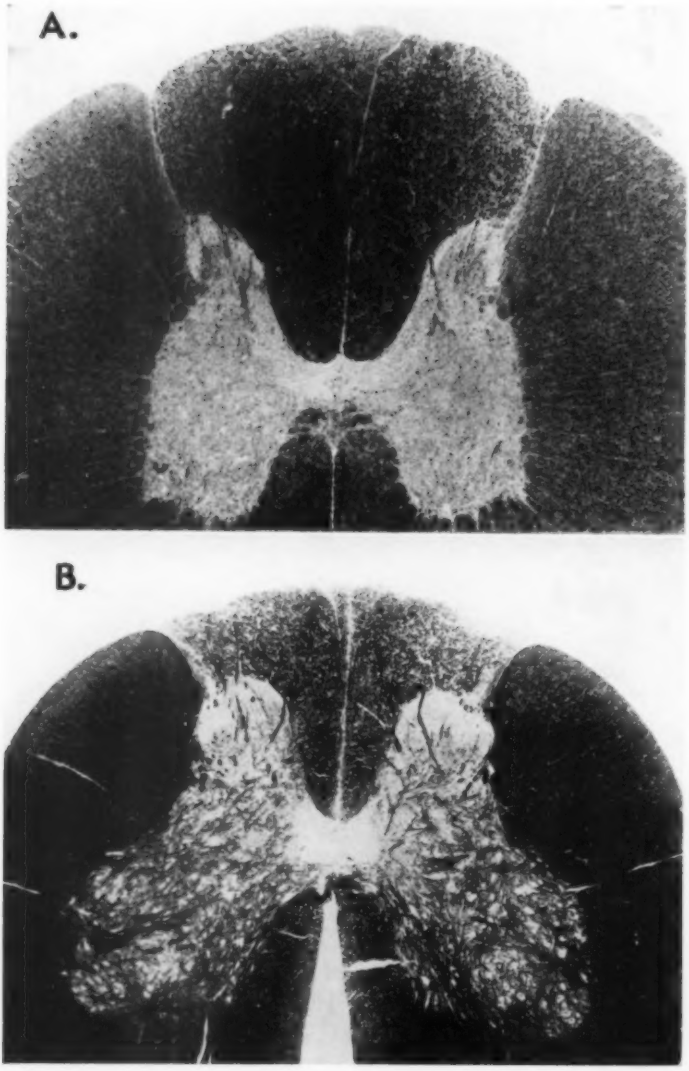

Fig. 2A Photomicrograph of transverse section of spinal cord of dog 9 showing very extensive (4+) destruction of myelin in posterior columns. Marchi stain. × 7.
Fig. 2B Extensive (3+) demyelination of posterior columns in spinal cord of dog 7. Kultschitzky stain. × 7.

nerve changes are usually more extensive than the cord lesions, it seems justifiable to assume that they precede the latter. It can also be shown quite clearly that the demyelinating process extends centrally to the cord along the posterior nerve roots, the degree of demyelination becoming greater, the longer the subsistence on the deficient diet.

The presence of minimal or at most mild demyelination in the control animals suggests that the basal diet may have been slightly deficient in some other factor besides riboflavin. An alternative explanation is that our dosage of 25 μg. of riboflavin daily was after all slightly inadequate. The first explanation is favored for the following reasons: In both experimental dog 1 and its control companion 2 the same mild degree of demyelination of peripheral nerves was observed after a period of only 152 and 158 days respectively, although the control animal was receiving 25 μg. of riboflavin per kilogram daily as compared with only a trace for the experimental dog. It is difficult to believe that such widely different intakes of riboflavin could have had the same effect on the nerves. On the other hand, if a moderate deficiency of some other factor in the basal diet is postulated, both animals might be expected to show the same effect.

Another reason for this belief is that rats given tikitiki plus the liberal dosage of 40 μg. riboflavin daily showed slight myelin degeneration. Some rats receiving tikitiki without riboflavin developed a more advanced yet still rather mild destruction of myelin. Because of the relatively mild form of the neuropathy in rats lacking riboflavin it seems highly improbable that rats receiving 40 μg. of riboflavin daily, a level sufficient to prevent all outward manifestations of riboflavin deficiency, should develop nerve defects caused by lack of this factor.

The liver. In view of the report of Sebrell and Onstott ('38) that riboflavin deficiency in dogs is characterized by extremely fatty ("yellow") livers, it is of interest to report our findings. The livers of all animals in this study were examined macroscopically at autopsy. Only three of these appeared abnormal.

The liver of dog 1 had a somewhat yellowish cast, while those of experimental dogs 9 and 53 were typical "yellow" fatty livers. Of five experimental dogs whose livers were examined histologically, three, dogs 13, 51, and 55 had normal livers, while those of dogs 7 and 53 were fatty but otherwise normal. Likewise, of three control animals examined, dogs 8, 10, and 52, all showed some increase in fat, but aside from this the microscopic appearance of the livers was normal. Thus, there was no consistent relationship of the condition of fatty livers to the riboflavin deficiency; both control and experimental animals exhibited this condition. It is pertinent to point out that our experiments were controlled by the use of inanition-control animals, whereas no controls are reported in the experiments of Sebrell and Onstott. It is indicated then, that the condition of fatty liver under discussion is related in some way to the partial inanition which cannot be entirely obviated when studying riboflavin deficiency, and not specifically to the lack of this factor. It is also possible, of course, that the differences in the two groups of observations are to be attributed to the differences in the basal rations employed.

Hemoglobin and erythrocyte levels in the blood. In our previous paper dealing with acute riboflavin deficiency it was reported that two out of three dogs developed lowered concentration of blood hemoglobin; the third animal gave normal values at all times. The anemia was characterized by little or no change in erythrocyte count, but a decrease in concentration of hemoglobin, the values for hemoglobin, which were 14 to 15 gm. per 100 cc. in the beginning, dropping to the level of 9 to 10 gm. per 100 cc. in one animal, and to 7 to 8 gm. in the other. We are now able to report concerning four additional dogs exhibiting the chronic picture of riboflavin deficiency and their respective inanition controls. In contrast to the observation of moderate anemia in the animals exhibiting the acute deficiency, it is a striking fact that the four animals, dogs 7, 9, 13, and 51, kept in a state of chronic deficiency for from 208 to 540 days, maintained concentrations of hemoglobin from 12 to 18 gm. per 100 cc. of blood, as

determined by bi-weekly examinations. While the values for hemoglobin tended to fluctuate a little, there was no upward or downward trend with the progress of the experiment. The hemoglobin values of the four inanition control animals behaved similarly. From this it would appear that when there is only a moderate but insufficient supply of riboflavin to the body, the hemopoietic system is spared, in contrast to other systems. Thus these dogs, after over 500 days of subsistence on the diet with insufficient supply of riboflavin, although exhibiting neuromuscular, ocular, and other manifestations of abnormality, nevertheless were maintaining erythrocyte counts and hemoglobin concentrations that were within accepted normal ranges.

Gastric analyses. Near the end of the experiment tests were made of the gastric contents of three chronically-deficient dogs (nos. 7, 9 and 13) for free and combined acid, and all values were within what are commonly regarded as normal limits. Evidently a long-standing moderate shortage of riboflavin cannot be regarded as having any specific effect on the gastric secretory mechanism.

DISCUSSION

In our previous paper dealing with acute riboflavin deficiency it was pointed out that marked deprivation with respect to this factor is associated with the development of a profound collapse in from about 100 to 150 days, which condition can be treated successfully by prompt administration of the missing factor. Such treated animals fail to show any specific neurologic disorders. The present paper, dealing with what might be called chronic riboflavin deficiency, makes it clear that long-continued subsistence on a diet containing some, but less than the minimal amount of, riboflavin required for perfect health, is not associated with collapse. Nevertheless, such a dietary regimen does result eventually in definite changes in the nervous system, appearing first as a demyelination of peripheral nerves which later progresses to involve the posterior columns of the spinal cord.

The fact that definite neurologically abnormal behavior developed in the present study so much later (after from 475 to 505 days) than in the earlier studies of Zimmerman and Burack ('34) and Zimmerman, Cowgill and Fox ('37) suggests that the diets used in these earlier studies were to some extent deficient in some other heat-stable factor of the B complex besides riboflavin. This would mean that the combination of vitamin B_1 concentrate prepared from rice polishings (Block and Cowgill, '32) and liver extract 343 powder [7] as used in the 1937 study, lacked some other water-soluble B-complex factor besides the riboflavin which was the intended variable of interest. Another reason that may be offered in support of this belief is the fact that in the 1937 study most of the dogs developed a secondary anemia which is in contrast to the observations made in the present investigation.

The fact that muscular weakness eventually appeared in the present chronically deficient animals suggests that the weakness which characterized the animals of the 1934 study, and which caused considerable difficulty at the time in detecting the presence of a true ataxia, thus leading to the perfection of technics such as use of the rotating table, obstacle board, training to walk on hind legs, etc., was indeed an expression of the marked shortage of riboflavin.

It may be pointed out that the neurologic symptoms of riboflavin deficiency in the dog are very different from those in vitamin B_1 deficiency. In the latter condition one of the first noticeable effects is the development of spasticity which causes a striking abnormality of gait. In contrast to this, dogs on a diet low in riboflavin continue to exhibit a normal or nearly normal gait, even when degeneration of the nervous system has progressed to the point where the deep reflexes are lost.

In pigs subsisting on faulty diets Hughes ('39) has reported the development of paralysis which could be cured by the administration of whey concentrate. Riboflavin was believed to be the effective agent in the concentrate, but it is not entirely clear that lack of vitamin B_6 was not playing a role. So far

[7] Lilly.

as it goes, Hughes' report may be regarded as confirmed by our present paper.

In view of the contributions by Day, Darby and Langston ('37) and Bessey and Wolbach ('38) on the appearance of corneal changes in rats deprived of riboflavin, the observations reported in the present paper constitute an extension of this to another species, namely, the dog.

Axelrod, Lipton and Elvehjem ('40) suggest that earlier work on riboflavin deficiency reported from this laboratory (Street and Cowgill, '39) probably represented other deficiencies as well because some of the animals developed a hypochromic anemia. While it is true that some of the animals with acute riboflavin deficiency developed a mild anemia, none of the animals in the chronic deficiency showed any tendency whatever toward anemia (for illustrations, see fig. 1). This may be explained in this way, that complete dietary lack of riboflavin results in some interference with hemoglobin formation, whereas with a small but incomplete amount of this factor the blood-forming tissues are able to perform fairly effectively. If the anemia appearing in a few of the animals were due to some complicating deficiency other than that of riboflavin, the anemia should have developed to a greater degree in the chronic study, since the experimental periods were so much longer.

SUMMARY

Prolonged subsistence on a diet low in riboflavin has led to the development of neurologic abnormalities in dogs, as evidenced by clumsiness in hurdling obstacles and in treading a revolving table, and finally by loss of the deep reflexes of the limbs. This was accompanied by myelin degeneration of peripheral nerves and the posterior columns of the spinal cord, becoming more extensive with the length of the period on the deficient diet. The administration of crystalline riboflavin to inanition control dogs maintained them in apparently perfect health, as judged during life, and largely prevented degenerative changes in the nervous system.

An increased fat content of the cells in otherwise normal livers was observed irregularly in the animals fed the inadequate diet; it was seen also in the inanition control dogs. This suggests that these changes in the liver are to be attributed to the accompanying inanition rather than specifically to the lack of riboflavin.

In the dog, as in the rat, the appearance of opacities of the cornea may be one of the effects of deprivation of riboflavin.

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DEPENDENCE OF FETAL GROWTH AND STORAGE OF CALCIUM AND PHOSPHORUS ON THE PARATHYROID FUNCTION AND DIET OF PREGNANT RATS [1]

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(Received for publication February 21, 1941)

The work on which this report is based was planned with the object of determining the extent to which the composition of the rat fetus depends on the parathyroid function and intake of calcium and phosphorus of the maternal organism during pregnancy. To obtain accurate data of birth weights and composition, the newborn rats were either taken from the cages before there was an opportunity to nurse, or were removed from the uterus when there was unmistakable evidence that parturition had begun. The rat pups were wiped dry, weighed individually and a representative number was prepared for duplicate analyses. For each analysis two or more pups, weighing a total of approximately 10–12 gm., were each cut into several pieces, dried at 105°C. to constant weight in silica crucibles, then ashed in a muffle furnace. Calcium was determined by combining the method of McCrudden ('09–'10) with the titration procedure of Shohl and Pedley ('22). Phosphorus was determined by the method of Shohl and Brown ('32).

[1] This work was aided by generous grants from the Mead Johnson Company, Evansville, Indiana, and approved by the Council for Pediatric Research of the American Academy of Pediatrics.

RESULTS

The largest number of observations was made on diet no. 7 (Ca 0.49%, P 0.49%) of Cox and Imboden ('36 a). Forty litters from parathyroidectomized rats were compared with thirty litters from unoperated control animals. The differences between these two groups are shown in table 1.

Comparison of total fetal storage of calcium and phosphorus emphasizes the difference between fetal development in normal and parathyroidectomized animals. The pups from the parathyroprevic rats contained 30% less calcium than the control pups, while the fetal storage of calcium per litter was on an average less than half of that in the controls.

Rats fed diet no. 27 (Ca 0.122%, P 0.245%) of Cox and Imboden ('36 a) consumed during pregnancy approximately one-fourth of the calcium and one-half of the phosphorus ingested by the rats on diet no. 7. In spite of these differences, the offspring from both the control and parathyroidectomized rats on diet no. 27 were on an average almost identical in composition with those from the corresponding group fed diet no. 7. However, owing to the numerically larger litters produced by the controls on diet no. 27 than on diet no. 7, a result which is tentatively assumed to have been adventitious, the average fetal storage of calcium and phosphorus was actually higher in the former group than in the latter. On the other hand, in the parathyroid-deficient rats on diet no. 27 the litters were smaller than in the corresponding group on diet no. 7, a finding which may or may not have statistical significance, but which accounts, in part, for the difference in total fetal storage of calcium and phosphorus.

With diet no. 26 (Ca 0.017%, P 0.245%) it is practically impossible to breed parathyroidectomized rats successfully (Bodansky and Duff, '41 a). However, if before being placed on this diet such rats are fed diet no. 7 for a few days during the mating period, the proportion of pregnancies to matings is increased from about 3% to nearly 25%. In table 1 are summarized the data for ten litters from parathyroidectomized rats subjected to this routine, and, for comparison, ten

TABLE 1

Influence of parathyroid deficiency and mineral composition of the diet on fetal development and fetal storage of calcium and phosphorus

Diet number	7		27		26			16		19		8		10		12	
Condition of pregnant animals	N-C[1]	P-D[2]	N-C	P-D	N-C	N C[4]	P-D[4]	N-C	P-D	N-C	P-D	N-C	P-D	N-C	P-D	N-C	P-D
Number of litters	30	40	13	12	14	10	10	13	15	7	5	6	2	7	1	7	6
Average number per litter	9.3	8.6	11.5	6.4	9.5	10.0	7.6	8.2	8.4	10.7	9.2	11.5	7.5	10.0	9.0	9.9	8.8
Standard deviation	±3.1	±2.3	±2.3	±3.1	±1.5	±1.4	±3.9	±2.0	±2.8	±1.6	±2.5	±1.9	±0.7	±2.6		±2.3	±1.7
Average litter weights, in gm.	49.62	29.19	56.15	21.01	45.12	52.40	23.24	42.62	41.60	55.47	31.86	56.67	33.87	48.85	20.34	55.47	40.01
Standard deviation	±15.38	±8.20	±11.20	±6.45	±16.96	±8.02	±9.85	±9.67	±9.14	±7.67	±14.66	±9.43	±10.38	±12.97		±11.65	19.82
Average birth weight, in gm.	5.45	3.49	4.95	3.67	5.14	5.24	3.36	5.24	4.62	5.19	3.50	4.95	4.58	4.88	2.26	5.69	4.53
Standard deviation	±0.67	±0.83	±0.87	±1.11	±0.98	±0.30	±0.96	±0.49	±0.72	±0.36	±1.15	±0.67	±1.89	±0.23		±0.60	±0.59
Total solids, average %	13.51	12.93	13.49	12.88	13.41	13.17	12.75	12.92	12.94	13.37	13.21	13.60	13.91	13.48	14.05	13.85	13.10
Standard deviation	±0.54		±0.60	±0.83	±0.79	±0.22	±0.89	±0.52	±0.45	±0.20	±0.75	±0.52	±0.62	±0.58		±0.33	±0.78
Ash, %																	
Minimum	1.502	1.241	1.548	1.284	1.518	1.574	1.207	1.364	1.156	1.460	1.442	1.576	1.438	1.592		1.692	1.405
Maximum	1.962	1.770	1.838	1.734	1.852	1.732	1.590	1.650	1.618	1.712	1.586	1.826	1.444	1.770		1.854	1.680
Mean	1.675	1.474	1.678	1.470	1.701	1.663	1.411	1.503	1.479	1.639	1.518	1.710	1.441	1.662	1.450	1.777	1.547
Standard deviation	±0.108	±0.211	±0.106	±0.144	±0.100	±0.046	±0.142	±0.095	±0.131	±0.082	±0.056	±0.101	±0.004	±0.066		±0.063	±0.102
Calcium, %																	
Minimum	0.193	0.094	0.189	0.126	0.157	0.210	0.069	0.102	0.153	±0.162	0.101	0.197	0.144	0.211		0.218	0.172
Maximum	0.301	0.269	0.318	0.285	0.275	0.265	0.225	0.231	0.226	0.247	0.222	0.252	0.248	0.277		0.284	0.246
Mean	0.239	0.166	0.240	0.169	0.232	0.236	0.151	0.172	0.174	0.222	0.170	0.234	0.196	0.234	0.128	0.265	0.206
Standard deviation	±0.028	±0.049	±0.035	±0.049	±0.034	±0.018	±0.045	±0.038	±0.023	±0.028	±0.048	±0.021	±0.074	±0.027		±0.022	±0.030
Calcium, % ash, mean	14.22	11.21	14.24	11.37	13.62	14.20	10.55	11.43	11.79	13.48	11.19	13.66	13.60	14.07	8.81	14.88	13.23
Standard deviation	±1.05	±1.98	±1.38	±2.21	±1.56	±1.06	±0.43	±1.92	±1.24	±1.26	±3.01	±0.66	±5.09	±1.19		±1.04	±1.08
Phosphorus, %																	
Minimum	0.259	0.210	0.232	0.213	0.258	0.282	0.224	0.223	0.214	0.243	0.258	0.254	0.254	0.247		0.268	0.272
Maximum	0.336	0.338	0.343	0.342	0.346	0.308	0.301	0.295	0.280	0.304	0.308	0.335	0.285	0.352		0.329	0.287
Mean	0.295	0.271	0.291	0.273	0.296	0.292	0.263	0.253	0.248	0.281	0.279	0.289	0.269	0.284	0.231	0.297	0.277
Standard deviation	±0.012	±0.037	±0.039	±0.039	±0.023	±0.008	±0.025	±0.024	±0.020	±0.020	±0.018	±0.030	±0.021	±0.040		±0.020	±0.005
Phosphorus, % of ash, mean	17.64	18.34	17.31	18.54	17.41	17.58	18.64	16.84	16.89	17.15	18.42	16.91	18.65	17.08	15.96	16.71	17.98
Standard deviation	±0.99	±1.19	±1.94	±1.45	±0.91	±0.49	±0.72	±1.00	±1.63	±0.81	±1.23	±1.00	±1.42	±2.03		±1.94	±1.01
Calcium: Phosphorus ratio in ash[3]	0.81	0.61	0.82	0.62	0.78	0.81	0.57	0.68	0.70	0.79	0.61	0.81	0.73	0.82	0.55	0.89	0.74
Fetal storage, total, per litter:																	
Calcium, mean, in mg.	117.4	50.1	136.7	35.0	106.7	124.6	35.0	71.5	72.2	123.2	57.8	132.7	70.1	113.3	26.0	146.9	84.2
Standard deviation	±37.5	±22.6	±35.6	±12.5	±43.1	±26.9	±20.2	±16.8	±17.8	±25.6	±37.5	±27.0	±45.3	±29.0		±35.0	±31.5
Phosphorus, mean, in mg.	145.5	79.4	164.4	57.0	134.8	153.5	61.1	106.6	103.2	156.1	89.3	163.0	92.2	135.9	47.1	164.1	110.9
Standard deviation	±44.3	±22.1	±43.2	±17.7	±51.1	±27.0	±27.0	±19.2	±23.5	±25.7	±40.9	±22.5	±35.2	±30.3		±31.2	±26.8

[1] N-C denotes normal control rats. [2] P-D denotes parathyroidectomized rats. [3] Based on mean values for per cent calcium and phosphorus.

[4] Owing to the extremely low rate of reproductive success of parathyroid-deficient rats kept on diet no. 26 of Cox and Imboden, the procedure was adopted of mating these animals and a corresponding number of unoperated controls on diet no. 7, then transferring them to diet no. 26 on which they remained for the greater part (usually 14–20 days) of the gestation period.

litters from unoperated rats submitted, similarly, to a preliminary period (7 days) of feeding on diet no. 7. In addition, data are included for fourteen litters from unoperated rats which, during mating and pregnancy, were fed diet no. 26 exclusively.

Examination of the results for the control animals on diets nos. 26, 27, and 7, reveals that even severe calcium deprivation produced comparatively little change in the birth weight or composition (total solids, ash, calcium, and phosphorus) of the young. The total fetal storage of calcium and phosphorus fell within nearly the same range of variation on calcium-deficient diet no. 26, as on diet no. 7, which was presumably adequate.

On the other hand, the defects in fetal development in the parathyroidectomized rats, as far as these may be judged from chemical analysis of the newborn, were somewhat aggravated by the calcium deficiency of diet no. 26.

Cox and Imboden ('36 b) found that after several reproductive cycles, diet no. 16 (Ca 1.225%, P 0.245%) produced a lowered ash content in the offspring and a condition comparable to congenital rickets. These findings were confirmed in the present study, not only for second and subsequent litters, but for first litters as well. The newborn rats in this group yielded the lowest average values for total solids, calcium, phosphorus and Ca/P ratio.

The properties of diet no. 16 in offsetting the effects of parathyroid deficiency in the pregnant rat have been described in other connections (Bodansky and Duff, '41 a, b). Hypocalcemia, the associated tetany, and the high rates of maternal and fetal mortality are abolished. The bones of the parathyroidectomized rats undergo changes in weight comparable to those in the normal rats on the same diet, suggesting equivalent rates of demineralization and calcification. Finally, the composition of the young from parathyroprevic mothers fed diet no. 16 is the same as that for the corresponding controls (table 1). Fetal storage of calcium and phosphorus is practically identical.

The results may be interpreted as signifying that on this diet the mineral metabolism on the fetal side of the placental barrier is essentially the same in both groups of rats. Maternal parathyroid function, of primary importance when other diets are fed, is evidently not a significant factor in fetal development under these circumstances.

Diet no. 19 (Ca 1.225%, P 1.225%), containing the same high percentage of calcium as diet no. 16, but sufficiently more phosphorus to bring the Ca/P ratio down to a value of 1, differed from diet no. 16 in enabling the deposition of normal amounts of calcium and phosphorus in the fetuses of the control rats. The pups of the parathyroidectomized group were low in body weight, per cent ash, calcium, and in the Ca/P ratio of the ash. Total fetal storage of calcium and phosphorus was less than in the corresponding group on diet no. 16.

In normal rats fed diet no. 8 (Ca 0.49%, P 0.735%), fetal development was substantially the same as on diets 7 and 27. However, rats fed diet no. 12 (Ca 0.735%, P 0.49%) produced young which were somewhat heavier than the offspring in other diet groups and contained higher percentages of solids, ash, calcium, and phosphorus.

The pups from the parathyroidectomized rats on diets 8 and 12 were inferior to the controls on the corresponding diets. In relation to pups from other groups of parathyroidectomized rats they were somewhat superior. On diet no. 12, fetal storage of calcium and phosphorus attained values which were higher than those for any other group of parathyroidectomized animals.

Shelling ('32), and Richter and Eckert ('37), have shown that parathyroidectomized rats eat freely of diets rich in calcium and refuse diets rich in phosphorus, an observation confirmed by the present study. Diet no. 16, overabundant in calcium but low in phosphorus, was preferred to other diets by parathyroidectomized rats. Diet no. 19 was rejected by a few, but not by all animals, while diet no. 10 (Ca 0.49%, P 2.45%) was refused almost invariably. As a rule, the parathyroidectomized rats offered diet no. 10 lost weight rapidly

and, unless the ration was changed, death resulted from starvation.

Owing to these circumstances it was almost impossible to carry a parathyroidectomized rat through a successful pregnancy. Out of a group of more than twelve animals this was achieved only once. During the first month on diet no. 10 this animal lost nearly 80 gm. in weight. As a result of the third mating it became pregnant and regained approximately 50 gm. in weight during the period of gestation, which was somewhat prolonged. Tetany developed at term. Nine live pups were removed from the uterus. Analysis of maternal blood collected from the carotid artery during delivery gave the following results: serum Ca 3.2 mg. per 100 cc., P 5.25 mg. per 100 cc. The low weights of the pups and other data are given in table 1.

With most diets appetite was not significantly altered because of lack of parathyroid function. In general, the parathyroidectomized rat and its corresponding unoperated control consumed approximately the same quantities of food during pregnancy, as shown by the data in table 2. In some instances more food was consumed; in others somewhat less. Parathyroidectomized rat no. 526, fed diet no. 7 of Cox and Imboden, though consuming more food than rat no. 525, its control, nevertheless utilized for fetal development only about half of the calcium and phosphorus stored by the fetuses of the unoperated controls. This relationship held, not only for diet no. 7, but for certain other diets, as brought out by the data in table 2, which were typical of a larger number of similar balance experiments. These data provide conclusive evidence that on a given diet (7, 19, 26, 27) the differences in fetal mineral metabolism between normal and parathyroid-deficient rats cannot be attributed to quantitative differences in food consumption.

It is instructive to compare the utilization of calcium and phosphorus at various levels of intake. The data in table 2 for diets 26 and 27 reflect either a greater mineral depletion from the maternal organism, or a more economical utilization

TABLE 2

Showing that differences in the fetal storage of calcium and phosphorus between normal and parathyroidectomized rats fed a given diet were not due to differences in food consumption

RAT SERIAL NO.	DIET NO.	NORMAL CONTROL (N-C), OR PARATHYROIDECTOMIZED (P-D)	INCREASE IN WEIGHT DURING PREGNANCY	FOOD CONSUMED		CONSUMPTION OF		NUMBER OF PUPS	WEIGHT OF LITTER	FETAL STORAGE			
				Amount		Ca	P			Calcium		Phosphorus	
										Amount	Part of consumption	Amount	Part of consumption
			gm.	*gm.*	*days*	*gm.*	*gm.*		*gm.*	*mg.*	%	*mg.*	%
525	7	N-C	178–240	219	21	1.073	1.073	12	48.4	117.7	11.0	144.0	13.4
526		P-D	208–247	260	21	1.274	1.274	10	33.4	52.7	4.1	79.3	6.2
420	7	N-C	178–255	289	17	1.416	1.416	7	42.6	122.1	8.5	135.9	9.6
421		N-C	185–293	326	17	1.607	1.607	14	69.9	182.9	11.4	229.1	14.3
419		P-D	212–295	282	17	1.382	1.382	12	47.4	64.6	4.7	128.4	9.3
375	7	N-C	284–370	233	15	1.142	1.142	11	65.8	178.8	15.6	205.9	18.3
376		N-C	236–347	246	15	1.205	1.205	9	47.4	107.9	9.0	134.5	11.2
378		P D	222–290	226	15	1.107	1.107	8	31.0	78.8	7.1	99.5	9.0
397		P-D	177–224	214	15	1.049	1.049	11	36.2	64.3	6.1	103.5	9.9
387	27	N-C	277–306	243	18	0.297	0.596	9	51.9	126.8	42.7	138.1	23.2
383		P-D	208–260	204	18	0.254	0.510	9	31.0	51.8	20.8	92.0	18.1
393	27	N C	253–350	277	17	0.338	0.678	12	64.6	172.4	51.0	177.5	26.4
414		N-C	196–280	264	17	0.322	0.646	11	54.3	133.2	41.4	176.4	27.3
408		P-D	186–222	323	17	0.394	0.792	12	25.6	32.2	8.1	68.8	8.7
537 F_2	26	N-C	213–284	244	17	0.041	0.598	10	52.1	119.5	292.0	152.6	25.5
538		P-D	227–244	262	16	0.044	0.642	3	14.0	19.0	43.2	36.0	5.6
541		N-C	170–250	180	17	0.031	0.442	9	49.0	125.7	405.0	145.0	32.8
581		P D	221–255	234	17	0.040	0.574	8	33.1	57.7	144.0	91.6	16.0
350	16	N-C	200–243	196	17	2.400	0.480	6	36.0	75.3	3.1	105.5	21.9
349		P-D	186–257	184	17	2.250	0.451	8	42.8	70.3	3.1	103.5	23.0
567	19	N-C	160–248	217	16	2.660	2.660	10	49.7	112.8	4.2	139.8	5.3
566		P-D	212–255	233	16	2.860	2.860	9	29.9	66.4	2.3	92.0	3.2

of the calcium and phosphorus supplied in the rations. Studies now in progress indicate that both factors participate in varying degree, depending upon the composition of the maternal diet. When the diet is almost devoid of calcium, as is the case with diet no. 26, most of the calcium for fetal development is derived from the maternal bones. Very little is lost by excretion. A balance experiment performed on rat 537 during the first reproductive cycle gave the following results: calcium consumed during the last 18 days of pregnancy, 30 mg.; calcium excreted in feces and urine during the same period, 45 mg.; calcium contained in newborn pups, 116.4 mg.—equivalent to 387% of the calcium consumed during the last 18 days of pregnancy, or an estimated 320% of the amount consumed during the entire gestation period. The low excretion of calcium in rat 537 may be contrasted with an excretion of 639 mg. in another rat (no. 529), fed diet no. 7. On this diet, the rat consumed 900 mg. of calcium during the last 15 days of pregnancy. Fetal storage of calcium amounted to 109 mg. leaving a positive balance of 152 mg., representing the storage of calcium in the maternal organism.

In the parathyroidectomized rats the percentage utilization of dietary calcium for fetal development on a given diet was invariably much lower than in the corresponding controls, with the exception of those on diet 16. As shown by the data in table 2, the utilization, 3.14%, was identical for the parathyroidectomized rat and for its unoperated control.

Under conditions of extreme deficiency, as with diet no. 26, even the parathyroidectomized rats seemingly possessed some ability to mobilize calcium from the maternal reserves. Such mobilization was much less responsive to the needs of the organism and more variable than was the mobilization of bone salts in the animal with intact parathyroid function.

The data in table 3 for rats 421 (diet no. 7), 392 (diet no. 27) and 539 (diet no. 26), were selected for the reason that in each of these animals fetal storage of calcium attained the highest level for its respective diet group. A comparison of the three figures, 182.9 mg., 184.0 mg., and 182.8 mg., brings

out the remarkable fact that the fetal storage of calcium was nearly identical in these animals in spite of enormous differences in calcium intake during pregnancy. Here is conclusive evidence that in a young rat (no. 539, age 95 days) fed a diet nearly devoid of calcium, the requirements for fetal development were met from its own reserves. During pregnancy this rat gained 85 gm. in weight, of which 68.9 gm. represented the weight of the pups. The average birth weight, 5.75 gm., was

TABLE 3

Data selected to show that the fetal storage of calcium and phosphorus may attain approximately the same high levels in spite of large differences in the maternal intake of these elements during pregnancy

Rat serial number	421	392 [1]	539
Diet number	7	27	26
Age at onset of pregnancy	95 days	175 days	95 days
Change in weight during pregnancy, in gm.	185–285	250–338	225–310
Average weight of pups at birth, in gm.	5.0	6.3	5.7
Number of pups in litter	14	12	12
Weight of litter, in gm.	70.0	75.0	68.4
Consumption during last 18 days of pregnancy			
Calcium, in gm.	1.600	0.408	0.037
Phosphorus, in gm.	1.600	0.819	0.536
Fetal storage			
Calcium, in mg.	182.9	184.0	182.8
Per cent of consumption	11.4	45.4	494.0
Phosphorus, in mg.	229.1	222.8	211.9
Per cent of consumption	14.3	27.2	39.5

[1] This was a second litter; 421 and 539 were first litters.

relatively high. The total calcium content of the pups, 182.8, was equivalent to 494% of the calcium consumed during the last 18 days of pregnancy. Assuming, on the basis of other observations with diet no. 26, an excretion of about 50 mg. of calcium during the same period, the total loss of this element from the maternal organism amounted to about 200 mg., or an estimated 10% of its original reserve.

These relations account for the normal fetal development in a first pregnancy and, frequently, in a second pregnancy,

as well, but as the maternal calcium reserve is reduced, further loss during subsequent pregnancies is minimized, usually because of reduced fertility (fewer pups per litter). However, occasionally, fetal development and calcification are defective. These untoward effects may be obviated largely by providing the mother rat with an adequate diet for about a week between pregnancies. Thus, in one experiment in which a rat was fed diet no. 26 over a period of 5 months, but with 1-week intervals between pregnancies on diet no. 7, the fifth litter turned out to be best, consisting of fourteen pups, weighing 75.6 gm. and containing 163 mg. of calcium and 202 mg. of phosphorus.

DISCUSSION AND EXTENSION OF DATA

Excluding the consequences of repeated pregnancies in the face of long-continued dietary deficiency, the fact is that in the rat, as in man, fetal development and calcification may be normal under conditions of extreme calcium privation to the mother. Such independence for the fetus is, therefore, in marked contrast to the well-recognized dependence, postnatally, of the growing organism on its calcium intake (Sherman and Booher, '31; Fairbanks and Mitchell, '36; Cox and Imboden, '36 b; Boelter and Greenberg, '41; and others). It would seem, then, that in spite of marked variations and abnormalities in the nutrition and metabolism of the maternal organism, the mineral metabolism of the fetus may remain undisturbed. This condition, governed by regulatory mechanisms, may be included, properly, in the category of phenomena which Cannon ('29) designated by the term "physiological homeostasis". Maintenance of physiological homeostasis in the fetus when the maternal diet is deficient in calcium depends principally on a combination of two factors, the existence in the maternal organisms of a mobilizable calcium reserve and adequate stimulation of parathyroid activity.

Rachitogenic diet

These regulatory mechanisms evidently fail when the diet is disproportionately high in calcium. The rachitogenic prop-

erties of diets with a high Ca/P ratio are now well recognized, Bethke, Kick and Wilder ('32) having shown that increasing the ratio from 1.0 to 5.0 causes in rats a progressive decrease in growth, bone ash, and percentage of inorganic phosphorus in the blood serum. Similar observations have been recorded by Brown and co-workers ('32), Shohl and Wolbach ('36), and others, while Cox and Imboden ('36 b) found that such diets produce defective fetal calcification, an observation which has been confirmed and amplified in the present study.

As has been brought out, diet no. 16 (Ca/P = 5) promotes demineralization of the maternal skeleton, at the same time impairing fetal calcification. The data needed to explain the effects in the fetus on a quantitative basis are incomplete, but in anticipation of further investigation, their relation to the composition of the maternal blood, and, presumably, also to that of the fetal blood may be suggested. It is significant that in five out of six rats fed diet no. 16, the concentrations of inorganic phosphorus of the blood serum at term was 2.5 mg. per 100 cc., or less, values which are very low for the rat. The serum calcium averaged 11.54 mg. The Ca × P product for the group averaged 21.9, and was the lowest encountered for any normal group of rats.

In this connection it will be recalled that Howland and Kramer ('22) brought out the relation of the Ca × P product to calcification. They showed that in rickets the product was 35, or less, whereas normally the product was 40, or more. Logan ('40), in a review of the chemistry of calcification, notes that although the product can now be more accurately expressed in terms of $Ca^{++} \times HPO_4^{=}$, the original formulation of Howland and Kramer still retains its practical value.

If one resorts to this formula in order to explain the defective fetal calcification in rats fed diet no. 16, he may encounter several objections, one being that the only available analyses were those made at term; another is that the data pertain to the maternal and not to the fetal blood. However, from other evidence (Collip, '27; Needham, '31; Mull, '36) it may be assumed that the concentrations of calcium and phosphorus

on the fetal side of the placental barrier, though somewhat higher than on the maternal side, run roughly parallel. Evidently, this relation prevails even when one or both constituents are abnormally low in the maternal circulation, as illustrated by the following data recorded by Maxwell ('34) in a case of osteomalacia:

Maternal blood during delivery: Ca 5.4 mg., P 2.9 mg. per 100 cc.
Cord blood: Ca 8.1 mg., P 4.2 mg. per 100 cc.

Viewed in this light and also from the standpoint of Howland and Kramer's ('22) concept, the low Ca $\times$ P product in the rats fed diet no. 16 assumes significance. The product averaged 21.9, and in one instance was only 6.3, representing conditions which were incompatible with normal bone salt deposition. In rats fed other diets, the products were as follows (average values): diet no. 7, 42.5; diet no. 8, 53.4; diet no. 10, 49.1; diet no. 12, 52.9; diet no. 19, 42.6; diet no. 26, 31.9; diet no. 27, 47.0. These figures may be correlated with the finding that, out of all the diets employed in these studies, diet no. 16 stood out, alone, as the one which prevented normal fetal calcification in otherwise normal rats.

Maternal parathyroid deficiency

In parathyroidectomized rats, the principal mechanism for mobilizing calcium from the bones is abolished. Excretion of phosphorus is also impaired, as a consequence of which the concentration of this element in the blood tends to increase. Accordingly, even though the calcium is sharply reduced, the Ca $\times$ P product is not invariably low.

Correlation of the product with fetal calcification is, therefore, not as consistent as in the unoperated controls. Thus, for the parathyroidectomized rats fed diet no. 27, the product based on average values for calcium and phosphorus was $4.24 \times 10.76 = 45.7$, a figure theoretically compatible with normal calcification, though, actually the fetal storage of calcium and phosphorus was deficient. The explanation for this paradox may lie in the observation of McLean, Barnes

and Hastings ('35) that parathyroid deficiency causes a sharp reduction in Ca^{++} ions, and the related demonstration by McLean and Hinrichs ('38) that increasing the phosphate concentration in the blood promotes the formation of a colloidal calcium phosphate at the expense of calcium ions and calcium bound to protein.

For other diets, the Ca $\times$ P products in the parathyroidectomized rats were as follows (average values): diet no. 7, 36.4; diet no. 8, 27.2; diet no. 10, 16.8; diet no. 16, 21.8; diet no. 19, 32.1; diet no. 26, 22.1.

On the basis of the analytical data presented in this and previous papers it could be concluded that the fetal growth and calcification depend not so much on the calcium and phosphorus contained in the diet as on the levels of calcium and phosphorus maintained in the blood, normal levels of both in the mother and fetus promoting normal calcification, low levels of either preventing such calcification and inhibiting fetal growth generally.

Final proof of this would require analysis of the fetal blood and other data which, owing to technical limitations, are as yet unavailable. However, the indirect evidence is significant. On the basis of Kozelka, Hart and Bohstedt's report ('33) that in parathyroidectomized pregnant dogs the hypocalcemia and associated symptoms may be relieved by the administration of viosterol, 400 units of this vitamin were given daily during pregnancy to rats fed diet no. 7. While this did not produce a uniform effect on the serum calcium at term, the average for the group (5.4 $\pm$ 1.8 mg.) was somewhat elevated. This elevation, though moderate, was accompanied by definite improvement in fetal development. Analysis of thirteen litters from parathyroprevic mothers yielded the following average values and standard deviations from the mean: number of pups per litter 10.1 $\pm$ 1.7; birth weight 4.1 $\pm$ 0.7; ash 1.64 $\pm$ 0.12%; calcium 0.216 $\pm$ 0.031%; phosphorus 0.295 $\pm$ 0.022%; total fetal storage of calcium 88 $\pm$ 22 mg.; total fetal storage of phosphorus, 120 $\pm$ 29.8 mg.

Effect of aluminum

More remarkable than the effects produced by viosterol in parathyroidectomized pregnant rats were those obtained by incorporating 4% of aluminum acetate in diet no. 7. Jones ('36) found that aluminum acetate, or sulfate, protects rats from the symptoms which usually follow parathyroidectomy, the effect of the aluminum being related to reduction of the serum phosphorus and increase in serum calcium. Our results for pregnant rats agree with Jones' data for non-pregnant rats, as shown by the following values (averages and standard deviations) for eight parathyroidectomized animals at term: serum calcium 7.68 ± 1.90; phosphorus 4.23 ± 2.41. The serum calcium was considerably above, and the phosphorus considerably below, the corresponding values previously established for pregnant parathyroidectomized rats fed diet no. 7 (Ca 4.65 ± 1.06; P 7.81 ± 4.77; Bodansky and Duff, '41 a).

Analysis of the eight litters from these animals yielded the following average values: pups per litter, 10.3 ± 3.4; birth weight, 4.5 ± 0.7; ash, 1.59 ± 0.115%; calcium, 0.203 ± 0.026%; phosphorus, 0.254 ± 0.023%; total fetal storage of calcium, 93.8 ± 40 mg.; total fetal storage of phosphorus, 126.1 ± 46.0.

SUMMARY AND CONCLUSIONS

1. In rats with intact parathyroids fetal growth and storage of calcium and phosphorus may show little difference in spite of large differences in the maternal intake of these elements. A diet nearly devoid of calcium does not necessarily prevent normal fetal growth or alter the mineral composition of the offspring.

2. However, if the maternal diet contains an overabundance of calcium, so that the Ca/P ratio is abnormally high (diet no. 16 of Cox and Imboden, Ca 1.225%, P 0.245%, Ca/P ratio = 5), the deposition of bone salts in the embryo is definitely impaired.

3. Normal development of the fetus, including growth and calcification, depends on the parathyroid function of the

maternal organism. Lack of parathyroid secretion during pregnancy disturbs the mineral metabolism, not only on the maternal side, but also on the fetal side of the placental barrier.

4. The dominance of maternal parathyroid function as a factor in fetal development was abolished in rats fed rachitogenic diet no. 16 of Cox and Imboden. On this diet, parathyroidectomized and normal rats produced young which did not differ significantly in composition.

5. On a given diet, differences in the fetal storage of calcium and phosphorus between normal and parathyroidectomized rats were not due to differences in the amounts of food consumed during the period of gestation.

6. Administration of viosterol to parathyroidectomized rats fed certain diets produced a moderate increase in the average concentration of serum calcium and definite improvement in average fetal growth and fetal storage of calcium and phosphorus.

7. An even more conspicuous effect was obtained by incorporating 4% of aluminum acetate in the diet of the pregnant parathyroidectomized rats. Associated with the reduction of the serum inorganic phosphorus and increase in the serum calcium there was marked improvement in fetal growth and in fetal storage of calcium and phosphorus.

8. It is concluded that the development of the fetus and its ability to store calcium and phosphorus depend on the maintenance of suitable concentrations of calcium and phosphorus in the blood on the maternal side, and, presumably, also on the fetal side, of the placental barrier.

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THE GOITROGENICITY OF SOYBEANS [1]

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(Received for publication January 20, 1941)

In 1938, Patton, Wilgus, and Harshfield ('39) succeeded in producing goiter experimentally in chickens. The ration used in this work contained 25% of soybean oil meal as the chief protein supplement. About that time, Sharpless ('38) reported that a diet containing 75% of raw soybean flour induced in the thyroid of young rats an enlargement of three to five times normal, thus confirming the earlier evidence of McCarrison ('34) that soybeans would produce an enlargement of the thyroid of rats. It therefore appeared possible that the goiter observed in chickens was caused by the amount of soybean oil meal used, which was considerably in excess of that customarily employed. Hence, a study was undertaken to ascertain whether soybeans and soybean oil meal are goitrogenic to poultry and whether goiter can be produced without their presence in the diet.

EXPERIMENTAL

Single-comb white Leghorn chicks of a closely inbred strain were used in the experiments described in this paper. The chicks were hatched from hens receiving diets with known concentrations of iodine. The white rats used in one experiment were likewise closely inbred.

All experiments with chicks were conducted in battery brooders. Temperature in the battery room was maintained at 70° F. in winter and rarely went over 85° F. in summer.

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Lights were used to maintain a 14-hour day. The chicks were evenly distributed in the various pens. They were weighed individually each week. The rats were confined in individual cages with wire screen floors. At the end of the experimental period in experiment 1, representative chicks and rats were selected from each pen according to sex, weight, and condition. These animals were killed, and their thyroid glands were removed. The chick glands were trimmed and weighed immediately. Owing to the difficulty of accurate and quantitative removal, the rat glands were not weighed. The glands were fixed in Bouin's solution, sectioned, and stained by a special method to be described elsewhere.

The special staining method was developed by a series of modifications of Mallory's connective tissue stains, following the observation by one of us (F.X.G.) that the colloid of the thyroid follicles may be stained selectively red or blue, depending upon its density and possibly upon its high or low iodine content. A standard was evolved on the basis of which thyroid histology could be more closely correlated with thyroid function and with the nutritional status of the experimental animals kept on various diets. A systematic study of thyroid glands showed that they could be readily classified in ten distinct groups. Each group was assigned a mathematical score. Scores from 1 to 5 marked the range of goiter observed from a mild hyperplasia and hypertrophy to the diffuse type of severe struma. In the other direction the scores of minus 1 to minus 4 stood for thyroid glands of diminished activity ranging from slight inactivity to colloid goiter. In the latter group fall most of the glands of birds which received large amounts of iodine supplementary to their rations.

Standard errors [3] were calculated for body weight, thyroid weight, and histological score. In experiments 2 and 3, all surviving chicks were sacrificed for thyroid glands. The thy-

[3] Standard errors were calculated as follows: S.E. $= \sqrt{\frac{\Sigma W^2 - \Sigma WM}{N(N-1)}}$

The error of the average body weight for both sexes was calculated by the formula: $\sqrt{\frac{(S.E._1)^2 N_1 + (S.E._2)^2 N_2}{N_1 + N_2}}$

roid weight is expressed as milligrams of fresh thyroid per 100 gm. of body weight (Remington and Levine, '36). Since there was observed to be no relationship between body weight and goitrogenicity of the ration, growth data are omitted from the tables.

Iodine additions to the ration were made in the form of dilute alkaline, alcoholic solutions of KI. The amount of iodine was ascertained in each ration by a modification of the von Kolnitz and Remington method (Gassner, '40). Three experiments are reported here.

The basal rations used in these experiments were as follows:

	EXP. 1	EXP. 2 & 3
Ground yellow corn	51.5	54.0
Dried yeast residuals	5.0	7.5
Steamed bone meal	2.5	2.5
Vitamin D preparation [4]	0.05	0.05
Manganese sulphate	0.015	0.015
Sodium chloride	1.0	1.0
Total	60.065	65.065

The essential variables in each experiment are given in each table of results.

In experiment 1, eight pens, each containing twenty-five 1-day-old chicks, and a similar number of lots, each containing four male rats 28 days of age, were used. The chick experiment was conducted for 6 weeks and the rat experiment for 8 weeks. The following supplements were added to the basal ration: 35% of soybeans, ground fresh daily, or autoclaved; or 30% of expeller process soybean oil meal; or 28.7% of solvent process soybean oil meal; or 14% of casein and 1.5% of yeast. The supplements used in each ration are indicated in table 1. Five per cent of alfalfa leaf meal was added to each ration except in pen 6 in which it was replaced by $2\frac{1}{2}$% each of yeast and starch. Corn starch was used to make a total of

[4] This was a solution of vitamin D containing 1,000 A.O.A.C. units per gram, prepared by diluting in Wesson oil a solution containing 50,000 units per gram. The active agent was designated "D-activated animal sterol in oil" by the manufacturer. We are indebted to Dr. J. J. Waddell of Du Pont de Nemours, Inc., for this material.

100 parts. The yeast variations were made in order that the calculated vitamin B_2 (G) content of all rations would be approximately the same. The soybean oil obtained by chloroform extraction and used in pen 8 replaced the same amount of corn starch. This extraction was done at room temperature, and the solvent was removed by low-vacuum distillation.

In experiment 2, thirteen pens, each containing twelve 10-day-old chicks, were used. These chicks had been depleted of their iodine reserves by holding them on the low-iodine basal ration from time of hatching. The duration of the experiment

TABLE 1
Experiment 1

PEN NO.	RATION	IODINE	CHICK THYROIDS			RAT THYROIDS	
			No.	Weight	Histology	No.	Histology[1]
		μg./kg.		*mg./100 gm.*			
1	Practical	313	6	7± 0.7	0.0±0.0	2	0.0±0.0
2	35% soybeans	94	6	69± 8.1	3.8±0.1	8	2.6±0.3
3	35% soybeans, autoclaved	94	6	22± 3.8	2.2±0.2	2	1.8±0.3
4	28.7% soybean oil meal, solvent, A	81	7	62±17.2	3.7±0.2	2	2.5±0.5
5	30% soybean oil meal, expeller, B	81	7	46±10.4	2.5±0.3	2	2.5±0.0
6	Pen 5 ration but minus 5% alfalfa	25	6	149±24.0	5.3±0.2	5	3.7±0.3
7	14% casein	94	6	14± 1.4	1.3±0.3	2	1.0±0.0
8	14% casein plus 6% chloroform extract	63	6	9± 1.6	1.2±0.2	2	0.5±0.0

[1] According to the numerical scale devised. See text.

was 32 days. In experiment 3, nineteen pens were used. The first ten pens constituted a repetition of experiment 2 and the identical rations were used. These pens contained eleven 1-day-old chicks each. Each of the other pens contained twenty-five 1-day-old chicks. The duration of this experiment was 4 weeks. The additions of soybeans, soybean oil meal, and casein to the basal ration in experiments 2 and 3 were made in the same manner as in experiment 1 except in the case of pen 20. In this pen the soybean oil meal was doubled to 60%. This additional soybean oil meal replaced 25% of ground yellow corn and 5% of starch. A carotene solution was added to

approximate the vitamin A potency of the amount of yellow corn replaced. The soybean extracts replaced an equal amount of corn starch on the dry-weight basis, using casein as the protein supplement. The soybean residues acted as protein carriers. Corn starch was used as filler. In experiment 2, a control pen was included, the chicks in this pen being fed a practical starting mash recommended by this station for farm use.

The extractions of the soybeans were carried out at room temperature in a continuous extractor, acetone, ethanol, and ethyl ether being used in succession. It was necessary to heat the extracts to slightly above the boiling points of the solvents in order to remove all but a trace of the solvents. Final traces were removed by mixing with the ration and spreading the mixture out to dry.

Unpublished data have shown that thyroid weight per 100 gm. of body weight and thyroid histology in chicks on the same ration are essentially the same at 4 and 6 weeks of age. Since such data from duplicated pens in experiments 2 and 3 were in agreement they were combined and are so reported in table 2.

The intense goitrogenicity of soybeans is evident in all experiments (pens 2 and 10). The enlargement of the thyroid glands was from five to six times that obtained with the casein ration (pens 7, 11, and 21) at the same iodine level and was from eight to ten times normal (pens 1 and 9). Sharpless, Pearsons and Prato ('39), using rats, obtained enlargement about four times normal. Mild goiter occurred on the casein ration, the production of goiter in chicks without the use of soybean oil meal being thus demonstrated. Expeller process soybean oil meal produced less severe goiter than did soybeans (pens 5 and 19), the enlargement being about four times that produced with casein. This indicates that the goitrogenic factor is heat-labile. Histologically, the rats in experiment 1 did not show as severe goiter as did the chicks, but the results with each were in essential agreement. It appears that the chick thyroid is more sensitive to goitrogenic rations than is that of the rat.

Attempts to extract the goitrogenic factor with various fat solvents met with no success. The soybean oil obtained by chloroform extraction in experiment 1 (pen 8) lowered the thyroid weight, but the microscopic structure in the chick thyroids was not altered. The severity of goiter in rats was slightly lessened by this oil. Further attempts to remove the

TABLE 2

Experiments 2 and 3

EXP. NO.	PEN NO.	RATION	RATION IODINE	THYROID		
				Nos.	Weight	Histology[1]
			μg./kg.		*mg./100 gm.*	
2	9	Practical ration + iodine	18,000	6	12± 2.0	—1.0±0.0
2, 3	10	35% soybeans	38	12	100±18.2	3.3±0.16
2, 3	11	15% casein	25	19	16± 1.2	2.2±0.13
2, 3	12	3.9% acetone extract	31	18	16± 2.2	2.0±0.14
2, 3	13	1.2% alcohol extract	31	16	20± 2.1	2.0±0.17
2, 3	14	1.7% ether extract	31	13	19± 1.5	2.0±0.09
2, 3	15	28.0% acetone residue	13	10	79±13.8	3.6±0.15
2, 3	16	27.7% ethanol residue	13	16	139±23.9	3.1±0.31
2, 3	17	25.7% ether residue	25	13	77± 9.8	3.6±0.13
2, 3	18	Extracts + ether residue	31	14	65± 9.0	3.8±0.17
2, 3	19	30% soybean oil meal, expeller, C	19	37	59± 5.9	3.8±0.09
3	20	60% soybean oil meal, C	19	14	111±16.9	4.5±0.16
3	21	15% casein	15	23	21± 1.7	2.6±0.20
3	22	30% casein	12	19	19± 1.5	2.6±0.20
3	23	12% soybean oil meal, C, + 4% casein + 15% oat groats	19	9	74± 7.2	3.8±0.15
2, 3	24	Pen 19 ration + 5% alfalfa	38	17	44± 7.1	3.0±0.20
3	25	Pen 19 ration + iodine	56	6	26± 6.8	2.9±0.18
3	26	Pen 21 ration + 5% alfalfa	56	10	13± 1.3	2.3±0.23
3	27	Pen 21 ration + iodine	50	3	13± 2.2	0.5±0.20
2, 3	28	35% soybeans + iodine	18,000	15	12± 1.4	—0.3±0.13

[1] According to the numerical scale devised. See text.

goitrogenic factor by successive extractions with acetone, ethanol, and ethyl ether in experiments 2 and 3 failed to show any activity in the extracts (pens 11 to 14). The residues from the acetone and ether treatments (pens 15 and 17) differed only insignificantly in goitrogenicity from the original soybeans. The residue from the ethanol treatment (pen 16) produced significantly heavier goiters than did the other two

residues. This change in weight was not substantiated by the thyroid histology. It is of interest to note that all three extracts in experiments 2 and 3 significantly increased body weight.

The goitrogenic factor is somewhat heat-labile. Autoclaving the soybeans in experiment 1 caused a marked reduction in goitrogenicity. The expeller process oil meal was less goitrogenic than the unroasted solvent-process meal (pens 5 and 4). The latter, which is subjected to very mild heat in the extraction process, resembled the raw soybeans in ability to produce severe goiter. This has been confirmed in later work. Soybean oil meal prepared by the expeller process is subjected to considerable heat, whereas that prepared by the solvent process may have had much less heat treatment unless this is given as an additional step. For further details on this subject, reference may be made to the paper of Hayward ('36).

The data in table 2 (pens 19 and 20) demonstrate that the goitrogenic effect of soybean oil meal is quantitative at high levels. The histological examination of goiters produced by the ration containing 30% of soybean oil meal showed hypertrophy and diffuse hyperplasia of the follicular epithelium of such an extreme severity that doubling the amount of soybean oil meal to 60% could only slightly increase the degree of goiter, although it did practically double the parenchyma of the glands. The stroma of these glands was increased considerably, with a large number of parafollicular cells present. This effect is not due to an increase in protein level, since doubling the amount of casein had no effect (pens 21 and 22).

The effects of a modified goitrogenic ration (pen 23) indicate strongly that a ration containing 12% of soybean oil meal will produce as severe goiter as a ration containing 30%, even when casein is used as a protein supplement to equalize the protein level. Unpublished data indicate that oat groats have no effect on thyroid weight or histology in the chick.

An apparent antigoitrogenic action of alfalfa leaf meal was first noted in experiment 1 (pens 5 and 6). The alfalfa leaf meal contained a small amount of iodine, and the data from

pens 19 and 24–27 of experiments 2 and 3 show that the antigoitrogenic effect was probably caused by this iodine. The marked effect of the addition of even a small amount of iodine in lessening the severity of goiter is in agreement with the observations of Remington and Levine ('36) on rats.

The addition of sufficient iodine (pen 28) will entirely overcome the effect of the goitrogenic factor in soybeans on thyroid weight and histology. This iodine addition did not improve growth. Other observations have shown that lower amounts of iodine are also preventive when soybean oil meal is used. Experiments to discover the critical level of iodine supplementation will be reported later.

DISCUSSION

Our results with chicks and rats corroborate the observations of Sharpless, Pearsons and Prato ('39) with rats that soybeans are goitrogenic, that the goitrogenicity is partially inactivated by heat, and that iodine will counteract the effect on the thyroid gland. In addition, the goitrogenicity of commercial soybean oil meal is established.

The data reported in this paper do not support those of Sharpless and his co-workers regarding the solubility of this goitrogenic factor or factors. These workers, using thyroid weight and iodine content as criteria, found that acetone and ether extractions of the beans resulted in a significant lowering of the goitrogenicity of the residues. They did not test the extracts. In our experiments, using thyroid weight and histology as criteria, the residues from successive extractions of soybeans with acetone, ethanol, and ethyl ether were not significantly different in goitrogenicity from the original soybeans. Neither the extracts thus obtained nor a chloroform extract showed any goitrogenic activity. Special care was taken to guard against exposure of the soybeans and the residues to heat. The report of Sharpless and his co-workers does not mention such precautions.

As outlined previously, the soybeans were treated successively with acetone, ethanol, and ethyl ether. The failure of

the significantly larger goiters produced by the residue from ethanol treatment to show any significant differences histologically from those produced by the other two residues is noteworthy. If these results can be confirmed, it will seem possible either that there are two goitrogenic factors or that a single factor acts selectively and progressively upon two receptive components of the tissue of the thyroid gland, i.e., the follicular epithelium and the interstitial connective tissue.

Doubling the size of the goiters by increasing the soybean oil meal from 30% to 60% of the ration agrees with the similar findings of Sharpless and co-workers. The lack of response in the thyroid glands to increasing the protein content of the ration to the same extent with casein indicates that the increase in protein level in the ration is not responsible. It is difficult to explain why 12% of soybean oil meal was as effective as 30%.

The effectiveness of iodine in correcting goiter produced by soybeans in chicks without improving growth demonstrates that the goitrogenicity of soybeans does not explain the poor growth which the raw soybeans are generally known to afford. Soybean oil meal is one of the best protein supplements in animal and poultry feeding. Since no detrimental effects on growth or general well-being of growing chicks were noted in this study on rations containing as high as 30% of soybean oil meal and since the presence of a small amount of iodine corrects the goiter, it is the opinion of the authors that no changes in present recommendations on the practical use of soybean oil meal in chick rations are justified on the basis of this report.

SUMMARY

The observations of Sharpless, Pearsons and Prato that soybeans are goitrogenic, that this goitrogenicity is partially inactivated by heat, and that iodine will counteract the effect on the thyroid gland have been corroborated. The goitrogenic factor in soybeans was not extracted by chloroform nor by successive treatment with acetone, ethanol, and ethyl ether.

This is not in accord with the report of Sharpless et al. This goitrogenic factor causes intense hyperplastic and hypertrophic changes in the thyroid glands of chicks and rats.

Soybean oil meal was found to be goitrogenic, but no detrimental effect other than that on the thyroid gland was noted in growing chickens.

Goiter was produced in chicks without the use of soybeans or soybean oil meal.[5]

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[5] We are indebted to the Iodine Educational Bureau, Inc., New York, for the establishment of an Investigatorship at the Colorado State College Experiment Station, from which part of the expense of conducting this project was obtained.

MAGNESIUM BALANCE STUDIES WITH INFANTS

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TWO FIGURES

(Received for publication January 16, 1941)

Magnesium is an essential element which exhibits a complex physiologic function. The major portion of the magnesium occurs in the skeleton where it constitutes one-fortieth to one-fiftieth of the calcium content. Magnesium is considerably more abundant than calcium in the soft tissues where, among other functions, it is an activator of the phosphatase and glycolytic enzyme systems and a depressor of muscular and neural irritability. For a discussion of magnesium metabolism the reader is referred to several recent reviews with extensive bibliographies (Schmidt and Greenberg, '35; Greenberg, '39; Duckworth, '39, and Stearns, '39).

Although it is improbable that the average mixed diet containing leafy vegetables is deficient in magnesium, the milk diets of infants during the first few months of life may contain less than optimal amounts of this element. Human milk has the low magnesium content of 3 to 6 mg. per cent whereas cow's milk contains 13 to 19 mg. per cent.

Two studies on magnesium balances in infants have been reported in the literature. Swanson ('32) presents data on three normal full-term infants who received breast milk and cow's milk with and without cod liver oil. With intakes of 5.4, 7.6, 11.2 and 10.4 mg. per kilogram of body weight per day there were retentions of 1.5, 1.5, 1.6 and 2.8 mg. per kilogram per day respectively. Urinary excretion of magnesium was small and the major portion of this element was lost through

the feces. Schlutz, Morse and Oldham ('33) published data from this laboratory on four infants followed through thirteen to eighteen metabolic balance periods. They had received powdered whole milk to which was added in some periods spinach, cellu-flour or artificial salt mixture. The magnesium intakes of the four infants averaged 15.5, 14.5, 14.2 and 13.7 mg. per kilogram per day with corresponding retentions of 1.2, 1.4, 0.8 and 0.2 mg. per kilogram. These investigators concluded, "In our experiments the retentions of magnesium have been small and very variable. . . . By far the greater amounts of magnesium were eliminated in the feces and in general the amount in the feces paralleled the total output of feces."

Further work by Swanson on the magnesium content of the fetus and on the composition of growth seems important. Iob and Swanson ('34) found 783 mg. of magnesium (286 mg. per kilogram of body weight) in one full-term fetus. This value is comparable to the data on the average magnesium content of forty-six fetuses reviewed by Macy and Hunscher ('34). Although there were some exceptions in the individual analyses, these authors found a fairly even increase in the total amount of magnesium retained from 83 mg. by the fourth lunar month to 784 mg. by the tenth lunar month. Givens and Macy ('33) had stated that the average magnesium content of the fetus at term was approximately 0.5 gm. In studying the composition of growth Swanson found that magnesium constituted 218 and 378 mg. per kilogram gain in weight when two infants received breast milk, but magnesium represented 802 and 582 mg. per kilogram of gain when the infants were on cow's milk formulas. Swanson stated that "the retentions of calcium and phosphorus in two full-term infants per kilogram gain in weight are much less than the amounts of these minerals found per kilogram of body weight for full-term infants at birth. In respect to magnesium it is quite the opposite in that the retentions per unit of growth exceed the concentrations per unit of body weight at birth." The above work would indicate the need for a constant adequate supply of magnesium for satisfactory growth.

The present study is reported in the hope that the data may be of value in determining the level of magnesium intake which will yield optimum retentions of this element for the infant. A series of metabolism studies have been conducted on each of nine male infants during the first 6 months of their lives. The infants were given milk formulas to which either corn syrup or honey was added with vitamin and mineral supplements. The infants remained in good health and the diets were adequate for satisfactory gains in weight. A more detailed description of the infants and technics is presented elsewhere (Knott, Shukers and Schlutz).

Each infant was studied for at least eight metabolism periods during each of which urine and feces were quantitatively collected for 5 consecutive days. Rest periods of 3 to 7 days intervened between the collection periods. After each change of diet an interval of several days was allowed to permit equilibrium before a new metabolism period was started. The urine was preserved by toluene and refrigeration, enough concentrated hydrochloric acid being added to make the urine acid to methyl red. Feces were marked off by carmine, preserved by acid alcohol (5% glacial acetic acid in 95% ethyl alcohol) and by refrigeration during the collection periods, and finally dried to constant weight before analysis. In addition to magnesium retentions, calcium and thiamine balances were determined during the same periods. Magnesium analyses were made according to the method of Fiske and Logan ('34) on filtrates from the calcium determinations.

During the eighty-two metabolic balance periods for which data appear in table 1, total magnesium intakes varied from 54.6 to 245.3 mg. per day with fecal losses amounting to 39.5 to 191.6 mg. per day, and urinary excretion of 1.0 to 21.6 mg. per day. Retentions were similarly variable within the limits of —23.5 and 105.9 mg. per day. The relation of the retention of magnesium to intake has been briefly summarized in table 2. During twenty-three periods with intakes of 11.8 to 15.0 mg. per kilogram per day, there were sixteen positive and seven negative balances with a mean positive retention of 1.11 mg.

TABLE 1

Daily magnesium balance in infants

SUBJECT PERIOD		WT.	FOOD					URINARY MAGNESIUM IN MG. PER		FECAL MAGNESIUM IN MG. PER		RETENTION IN MG. PER	
			Milk	Carbohydrate	Vit. D	Magnesium in mg. per							
		kg.			*I.U.*[2]	*day*	*kg.*	*day*	*kg.*	*day*	*kg.*	*day*	*kg.*
No. 1 John	4	5.42	E.LA.[1]	Honey	170	86.1	15.4	2.3	0.4	56.0	9.5	27.9	5.0
	5	5.76	E.	"	"	96.1	16.3	9.9	1.7	61.0	10.4	25.2	4.3
	6	6.05	"	"	"	89.7	14.4	7.1	1.1	60.0	9.6	22.7	3.6
	7	6.43	"	"	"	93.9	14.2	9.5	1.4	60.7	10.2	23.7	3.6
	8	6.76	"	"	"	97.5	14.3	10.3	1.5	74.6	10.9	12.6	1.8
	9	6.99	"	"	"	83.3	11.8	8.9	1.3	68.5	9.7	5.8	0.8
	10	7.15	"	"	680	92.8	13.0	7.2	1.0	87.6	12.3	− 1.8	− 0.2
No. 2 Bart	3	5.27	D.	Honey	170	140.	26.6	8.	1.5	101.3	19.2	30.7	6.1
	5	5.82	"	Corn	"	154.	26.5	10.8	1.9	135.6	23.3	7.6	1.3
	6	6.16	"	syrup	"	168.	27.3	8.9	1.4	136.3	22.1	22.8	3.7
	7	6.35	"	Honey	"	168.	26.5	10.6	1.7	126.6	19.9	30.8	4.9
	8	6.39	"	"	"	168.	26.3	15.3	2.4	128.1	20.0	24.6	3.8
No. 3 Hal	1	3.64	E.	Honey	0	75.0	20.6	5.2	1.4	59.8	16.4	10.	2.7
	2	3.90	"	"	"	73.1	18.7	5.8	1.5	79.3	20.3	− 11.	− 2.8
	3	4.25	E.LA.	"	"	80.0	18.8	4.9	1.1	55.8	13.1	19.3	4.5
	4	4.52	"	"	1000	79.2	17.6	5.7	1.3	57.3	12.7	21.9	4.9
	5	4.78	"	"	"	79.7	16.7	5.3	1.1	64.4	13.5	10.0	2.1
	6	5.04	"	"	"	79.7	15.8	4.7	0.9	75.7	15.0	− 0.7	− 0.1
	7	5.55	D.	"	680	137.9	24.8	8.1	1.4	74.0	13.3	56.8	10.2
	8	5.80	"	"	"	139.2	24.0	5.8	1.0	115.2	19.9	18.2	3.1
	9	6.35	"	"	"	147.8	23.3	2.9	0.5	75.4	11.9	69.5	11.0
	10	6.63	"	"	"	159.9	24.2	8.3	1.3	123.7	18.7	27.9	4.2
	11	6.94	"	"	"	173.6	25.6	8.4	1.2	83.6	12.3	81.6	12.0
No. 4 Ned	1	3.69	Skim	Honey	170	169.5	45.9	9.6	2.6	96.9	26.3	63.0	17.1
	2	3.99	D.	"	"	126.	31.6	10.9	2.7	138.6	34.7	− 23.5	− 5.9
	3	4.45	"	"	"	140.	31.5	16.4	3.6	115.8	26.0	7.8	1.8
	4	4.82	"	Corn	"	140.	29.0	14.1	2.9	118.8	24.6	7.1	1.5
	5	5.10	"	syrup	"	154.	30.2	19.3	3.8	147.2	28.9	− 12.5	− 2.5
	6	5.44	"	"	"	154.	28.3	20.0	3.7	138.4	25.4	− 4.4	− 0.8
	7	5.68	"	"	"	168.	29.6	20.8	3.6	132.0	23.2	15.2	2.7
	8	5.88	"	Honey	"	168.	28.6	21.6	3.7	112.1	19.1	34.1	5.8
	9	6.10	"	"	"	182.	29.8	12.8	2.1	111.9	18.3	57.7	9.5
	10	6.36	"	"	"	182.	28.6	11.5	1.8	158.4	24.9	12.1	1.9
No. 5 Myles	1	4.10	Protein	Honey	170	150.1	36.6	9.7	2.4	88.7	21.6	51.6	12.6
	2	4.20	Skim	"	"	155.4	37.0	3.0	0.7	92.4	22.	44.4	13.4
	3	4.41	D.	"	"	126.	28.6	6.8	1.5	106.	24.0	13.2	3.0
	4	4.64	"	"	"	126.	27.2	7.5	1.6	108.	23.3	10.5	2.4
	5	4.80	"	Corn	"	126.	26.3	10.3	2.1	81.4	17.0	34.3	7.1
	6	5.01	"	syrup	"	140.	27.9	11.2	2.2	90.4	18.0	38.4	7.7
	7	5.47	"	"	"	140.0	25.6	13.4	2.4	90.4	16.5	36.2	6.6
	8	5.78	"	"	"	154.	26.6	11.4	2.0	132.1	22.9	10.5	1.8
	9	5.94	"	Honey	"	154.	25.9	14.7	2.5	110.1	18.5	29.2	4.9
	10	6.19	"	"	"	168.	27.1	17.1	2.8	115.9	18.7	35.0	5.7
	11	6.43	"	"	"	168.	26.1	14.6	2.3	134.7	20.9	18.7	2.9
No. 6 Jack	1	4.21	D.	Honey	100	133.	31.6	8.9	2.1	98.5	23.4	25.6	6.1
	2	4.52	"	"	"	133.	29.4	9.1	2.0	87.1	19.3	36.8	8.1
	3	4.78	"	"	"	147.	30.8	11.8	2.5	114.6	24.0	20.6	4.3
	4	5.03	"	"	"	161.	32.0	14.0	2.8	113.8	22.6	33.2	6.6
	5	5.46	"	"	200	175.	32.1	15.6	2.8	171.7	31.4	− 12.3	− 2.3
	6	5.64	"	"	"	175.	31.0	14.9	2.7	163.9	29.1	− 3.8	− 0.7
	7	5.97	Protein	"	"	237.5	39.8	18.8	3.1	126.9	21.2	91.8	15.4
	8	6.36	"	"	"	237.5	37.3	20.3	3.2	111.3	17.5	105.9	16.1
No. 7 Noel	1	6.30	D.	Honey	0	182.	28.9	12.4	2.0	135.0	21.4	34.6	5.5
	2	6.67	"	"	"	182.	27.3	11.0	1.6	191.6	28.7	− 20.6	− 3.1
	3	7.10	"	"	"	196.	27.6	11.0	1.5	164.0	23.1	21.0	3.0
	4	7.32	"	"	"	196.	26.8	15.6	2.1	151.0	20.6	29.4	4.0
	5	7.63	"	"	200	192.2	25.2	12.3	1.6	177.9	23.3	2.0	0.3
	6	7.55	"	"	"	180.3	23.9	7.9	1.1	169.1	22.4	3.3	0.4
	7	7.63	Protein	"	"	237.5	31.1	14.4	1.9	120.1	15.7	103.0	13.5
	8	7.84	"	"	"	245.3	31.3	14.5	1.9	126.3	16.1	104.5	13.3
No. 8 Guy	1	3.83	E.	Honey	170	54.6	14.3	1.6	0.4	43.2	11.3	9.8	2.6
	2	4.10	"	"	"	54.6	13.3	1.0	0.2	39.5	9.6	14.1	3.4
	3	4.44	E.LA.	Syrup	"	62.4	14.1	7.1	1.6	61.8	13.9	− 1.0	− 0.2
	4	4.76	"	Honey	"	66.3	13.9	6.7	1.4	51.1	10.7	8.5	1.8
	5	5.08	"	"	340	72.8	14.3	12.6	2.5	57.1	11.2	3.1	0.6
	6	5.32	"	Syrup	"	75.4	14.2	11.7	2.2	60.8	11.4	2.9	0.5
	7	5.66	"	Honey	500	78.0	13.8	13.0	2.3	48.7	8.6	16.3	2.9
	8	5.97	"	Corn	"	85.8	14.4	13.3	2.2	63.5	10.6	9.0	1.5
	9[3]	6.33	"	syrup	"	123.7	19.5	17.3	2.7	108.1	17.1	− 1.7	− 0.3
	10	6.74	"	"	170	88.4	13.1	16.7	2.5	64.7	9.6	21.2	3.1
	11	6.99	"	"	"	88.4	12.6	18.5	2.6	74.2	10.6	− 4.3	− 0.6
No. 9 Geoffery	1	4.22	E.	Honey	170	62.4	14.8	5.8	1.4	49.2	11.7	7.4	1.8
	2	4.26	"	"	"	62.4	14.6	6.0	1.4	39.6	9.3	16.8	3.9
	3	4.54	E.LA.	Syrup	"	70.2	15.5	10.7	2.4	45.1	9.9	14.4	3.2
	4	4.87	"	Honey	"	70.2	14.4	10.0	2.1	73.6	15.1	− 13.4	− 2.8
	5	5.15	"	"	340	75.4	14.6	13.9	2.7	58.0	11.3	3.5	0.7
	6	5.36	"	Syrup	"	75.4	14.1	16.0	3.0	60.6	11.3	− 1.2	− 0.2
	7	5.56	"	Honey	500	80.6	14.5	18.7	3.4	60.2	10.8	1.7	0.3
	8	5.83	"	Corn	"	91.0	15.6	15.9	2.7	71.6	12.3	3.5	0.6
	9[3]	6.12	"	syrup	"	128.9	21.1	17.9	2.9	133.4	21.8	− 22.4	− 3.7
	10	6.44	"	"	170	96.2	14.9	12.8	2.0	100.8	15.7	− 17.4	− 2.7
	11	6.79	"	"	680	96.2	14.2	11.3	1.7	89.4	13.2	− 4.5	− 0.7

[1] Abbreviations: E., evaporated milk. E.LA., evaporated lactic acid milk. D., dried half-skim milk.
[2] International Units. [3] Ten grams added wheat germ per day.

per kilogram per day. Intakes of 15 to 20 mg. per kilogram occurred in ten periods, three of which were negative. The mean positive balance was 2.14 mg. per kilogram per day. There was one negative balance during seven periods on intakes of 20 to 25 mg. per kilogram which exhibited a mean positive balance of 3.99 mg. per kilogram. A mean positive balance of 4.16 mg. per kilogram per day was found in twenty-seven periods, two of which were negative, with intakes of

TABLE 2

Relation of retention of magnesium to level of intake

INTAKE		NO.	MEAN RETENTION	TOTAL	MEAN RETENTION	NO.	MEAN RETENTION
mg./kg./day			*mg./kg./day*		*mg./kg./day*		*mg./kg./day*
Less than 15	Positive	16	2.06	23	1.11		
	Negative	7	—1.06			33	1.42
15–20	Positive	7	3.51	10	2.14		
	Negative	3	—1.07				
20–25	Positive	6	5.27	7	3.99		
	Negative	1	—3.70				
25–30	Positive	25	4.69	27	4.16	44	3.96
	Negative	2	—2.45				
30–35	Positive	6	7.60	10	3.42		
	Negative	4	—2.85				
More than 35	Positive	5	14.92	5	14.92		
	Negative	0					

25 to 30 mg. per kilogram. At levels of 30 to 35 mg. per kilogram there were six positive and four negative balances with a mean retention of 3.42 mg. per kilogram per day. Five positive balances with a mean of 14.92 mg. per kilogram were found in the five periods with intakes over 35 mg. per kilogram per day. In brief, thirty-three periods at levels of intake under 20 mg. per kilogram per day exhibited a mean retention of 1.42 mg. per kilogram, whereas forty-four periods with intakes between 20 and 35 mg. per kilogram gave a mean retention of 3.96 mg. per kilogram per day. Seven of the nine infants had one or more periods with negative balances. In several instances these negative balances occurred on rela-

tively high intakes but there were proportionately twice as many negative balances on intakes under 20 mg. per kilogram as occurred when more magnesium was ingested.

The fecal excretion of magnesium was equivalent to approximately 80% of the intake. In six periods the fecal losses exceeded the intake and only in nine periods was it less than 60% of the intake. This result is in accord with the findings of other investigators.

The urinary excretion of magnesium varied from 1.0 to 21.6 mg. per day with a mean of 10.5 mg. per day. In general the magnitude of both fecal and urinary losses varied directly with the intake although there were numerous exceptions.

Retentions were slightly greater when honey was the source of added carbohydrate than when corn syrup was used for this purpose. The results are summarized in table 3. With powdered half skim milk furnishing the magnesium, thirty periods on honey averaged a total retention of 24 mg. as compared to ten periods on corn syrup with an average retention of 16 mg. per day. When evaporated lactic acid milk was fed, thirteen honey periods had a mean total retention of 6 mg. in contrast to a mean retention of -0.4 mg. per day for twelve corn syrup periods.

In table 3 the results are also summarized in relation to the vitamin D intake. A range of vitamin D levels from 0 to 1000 international units per day apparently had no consistent effect upon the retention of magnesium by the different infants. No correlations were observed between the level of vitamin D administered and the amount of magnesium excreted in the feces.

Skim milk and protein milk were the source of magnesium in seven of the periods which are reported in table 3. Intakes were over 150 mg. per day and 30 mg. per kilogram. Retentions were of large magnitude constituting one-third or more of the intake. This may be interpreted as indicating either better absorption of magnesium from these types of milk supplemented with honey or decreased intestinal excretion during these periods.

TABLE 3

Magnesium balances in relation to vitamin D intake, sources of milk and carbohydrate

TYPE OF MILK	ADDED CARBOHYDRATE	VITAMIN D INTAKE	NUMBER OF			DAILY MAGNESIUM BALANCE				
			Subjects	Periods	Negative balances		Intake total	Fecal per cent of intake	Urine total	Retention total
Skim	Honey	170	2	2	0		163	58	7	54
Powdered protein	Honey	170	1	1	0		150	59	10	52
	"	200	2	4	0		240	50	17	101
"		(total)	3	5	0	(mean)	222	52	16	92
Powdered half-skim	Honey	0	1	4	1		189	85	13	16
	"	100	1	4	0		144	74	11	29
	"	170	3	13	1		154	80	13	22
	"	200	2	4	2		181	95	15	−3
	"	680	1	5	0		152	62	7	51
		(total)	6	30	4	(mean)	161	78	12	24
	Corn syrup	170	3	10	2		150	80	14	16
"	(grand total)		7	40	6	(mean)	158	78	12	19
Evaporated	Honey	170	3	9	0		77	72	7	15
	"	680	1	1	1		93	94	7	−2
		(total)	4	10	1	(mean)	79	74	7	13
Evap. lactic acid	Honey	0	1	3	1		76	87	5	6
	"	170	3	3	1		74	82	6	1
	"	340	2	2	0		74	78	14	4
	"	500	2	2	0		80	69	16	9
	"	1000	1	3	1		80	82	5	10
		(total)	4	13	3	(mean)	77	80	8	6
	Corn syrup	170	2	5	3		81	83	13	5
		340	2	2	1		75	81	14	1
	"	500	2	4	2		107	86	16	−3
	"	680	1	1	1		96	93	11	−5
		(total)	2	12	7	(mean)	90	85	14	−0.4
"	(grand total)		4	25	10	(mean)	83	82	11	3
Evaporated milks	(grand total)		4	35	11	(mean)	82	80	10	6

When powdered half skim milk was a source of magnesium there were six negative balances out of forty periods. Mean intakes varied from 150 to 189 mg. (24 to 31 mg. per kilogram per day), fecal losses averaged 78% of the intake, and the mean retention was 3.72 mg. per kilogram per day.

When evaporated milk or evaporated lactic acid milk was the source of magnesium, with one exception mean intakes were less than 100 mg. per day. Eleven out of thirty-five balances were negative and the mean retention was 1.33 mg. per kilogram per day. The data do not indicate whether the type of milk or the level of intake is the controlling factor for these low retentions, but there is some evidence that the latter is more important. The summary of magnesium balances presented in figure 1 illustrates the probability that the magnesium content of the milk is a controlling factor in determining the magnitude of retentions.

It is interesting to contrast the behavior of subject no. 3 (Hal) on two types of milk. On powdered half-skim milk with an intake of 24.4 mg. per kilogram the retention was 8.1 mg. per kilogram, while evaporated lactic acid milk provided 18.8 mg. per kilogram but the retention was only 1.9 mg. per kilogram. The urinary excretion was unchanged. The fecal loss of magnesium was apparently the controlling factor in this instance since it constituted 62% of the intake on the first formula and 82% of the intake on the evaporated lactic acid milk formula. Fecal losses were also significant in period 9 for infants no. 8 and no. 9 (Guy and Geoffery) when 10 gm. of wheat germ were fed as a daily supplement. For these two periods the average daily magnesium of the feces represented 87 and 104% of the intake.

No correlation was found between the phosphorus loss in the feces and the fecal magnesium loss. There was also no apparent relation between the fecal phosphorus and the magnesium retention. The ratio of calcium to magnesium in the milk formulas varied from 12.5 : 1 to 7 : 1 with a mean of 10 : 1.

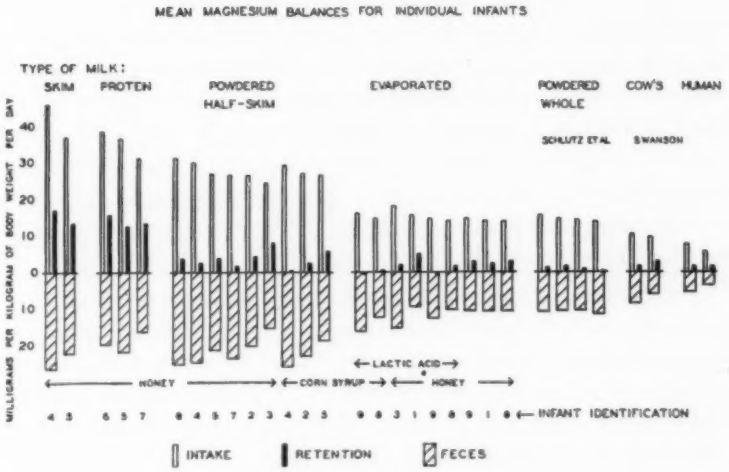

Figure 1

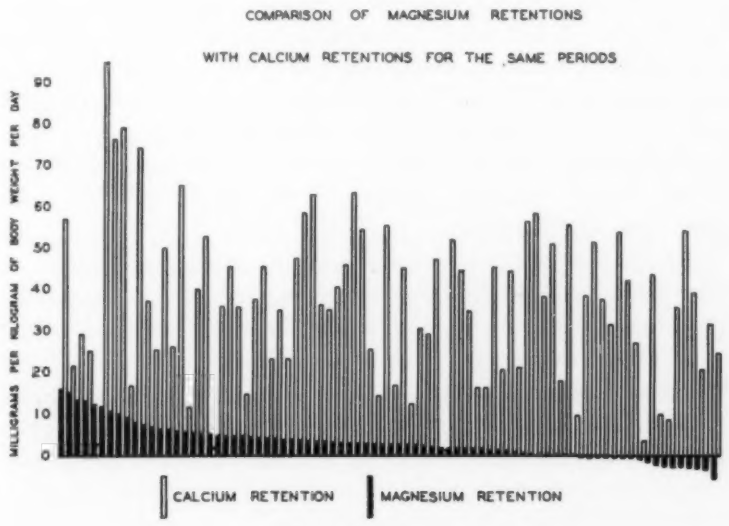

Figure 2

The ratio of calcium stored to magnesium retained was quite variable, but the mean of 10 : 1 is comparable to the ratio found in pre-school children by Daniels and Everson ('36). Although there is a tendency for calcium retentions to become less as the magnesium retentions diminish (see fig. 2) there is no definite correlation between them. These findings support the conclusion that calcium, magnesium and phosphorus, although related in metabolism, respond differently to the diverse factors acting upon them.

Magnesium retentions of approximately the same magnitude on comparable intakes were reported in pre-school children by Daniels and Everson as were found in this study with infants. The statement is made by Schmidt and Greenberg that from one-fifth to one-half of the magnesium excreted by infants and adults appears in the urine. This was true for the pre-school children of Daniels and Everson, but the infants of this investigation as well as of other studies (Swanson; Schlutz, Morse and Oldham) have excreted only one-tenth to one-fifth of their total magnesium loss in the urine. Thus it appears from these data that the criterion for adequate magnesium intake by pre-school children, suggested by Daniels and Everson, i.e., urinary loss of more than 3.4 mg. per kilogram, does not hold for infants since urinary magnesium levels of less than 2 mg. per kilogram were associated with good retentions of magnesium.

The data of this study show that higher magnesium intakes tend to increase magnesium retentions, but the present state of our knowledge is not adequate to determine whether or not these higher retentions have a pertinent effect upon the infant's well-being. Further work is necessary to establish an optimal intake and also to prove the possible existence of a magnesium deficiency disease in infants. Further investigation is also necessary to determine which factors that influence magnesium metabolism are responsible for the extensive variability in results.

SUMMARY

Infants from 2 to 6 months of age may be maintained in small but definite positive magnesium balance on diets containing 10 to 20 mg. per kilogram of body weight per day. Retentions are of greater magnitude when the intake is more than 20 mg. per kilogram per day.

No definite relationship was noted: (1) between magnesium and calcium retentions although there was a slight tendency for the retention of both substances to vary in the same direction; (2) between the fecal phosphorus excretion and the magnesium content of the feces or the magnesium retention; and (3) between the level of vitamin D and the magnitude of magnesium retention.

Although the data are not conclusive, slightly better retentions were found when honey was the source of carbohydrate than were observed when corn syrup was added. The variations noted for the different types of milk used can probably be ascribed to the level of magnesium intake.

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NUTRITIVE PROPERTIES OF STEAM-RENDERED LARD AND HYDROGENATED COTTONSEED OIL

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(Received for publication December 2, 1940)

Lard and hydrogenated cottonseed oil are among the most important fats in use for shortening and cooking purposes in the United States. The relative merits of these fats for culinary purposes have been studied extensively but less attention has been given to their nutritive properties. The purpose of the experiments herein reported was to determine the relative growth-promoting properties and digestibility of steam-rendered lard and hydrogenated cottonseed oil when fed to young rats.

In a previous publication the writers ('40) discussed the results of earlier experiments concerning the nutritive properties of lard and hydrogenated cottonseed oil. They also reported the results of experiments which they had conducted with young rats with refined steam lard and a well-known brand of hydrogenated cottonseed oil. The two fats had approximately the same growth-promoting value when fat constituted 5% of the diet, but the lard was superior when fat constituted 30 or 55% of the diet. The refined lard was superior in digestibility to the hydrogenated cottonseed oil at each level of intake. The digestive coefficients for the two fats were 97.6 and 88.2% at the 5% level, 96.5 and 92.2% at the 30% level, and 95.1 and 93.7%, respectively, at the 55% level. The present experiments furnish additional information concerning the nutritive properties of lard and hydrogenated cottonseed oil and show the relative efficiency with which each fat was utilized at four levels of intake.

MATERIAL AND METHODS

The lard and the hydrogenated cottonseed oil used in the present experiments were obtained from the same source. Only limited information concerning the manufacture of these products was supplied. The lard was steam rendered under 50 pounds pressure, settled, filtered, chilled, and packed in containers. It was not bleached. Refined cottonseed oil was hydrogenated to an iodine number between 70 and 75 and approximately 2% of fully hydrogenated oil was added. When

TABLE 1

Formulas for diets fed to rats

DIET NO.	TYPE OF SHORTENING	FAT IN DIET	CASEIN	SALTS	DEXTROSE	YEAST VITAMINS	COD LIVER OIL CONCENTRATE
		%	%	%	%	%	*cc. per kilo. of diet*
1	No fat	0	18.6	4.0	75.4	2.0	100
2	Steam-rendered lard	5	20.0	4.3	68.7	2.0	100
3	Hydrogenated cottonseed oil	5	20.0	4.3	68.7	2.0	100
4	Steam-rendered lard	15	23.0	5.0	54.5	2.5	100
5	Hydrogenated cottonseed oil	15	23.0	5.0	54.5	2.5	100
6	Steam-rendered lard	30	27.8	5.8	33.4	3.0	100
7	Hydrogenated cottonseed oil	30	27.8	5.8	33.4	3.0	100
8	Steam-rendered lard	54.2	34.8	7.3	0	3.7	100
9	Hydrogenated cottonseed oil	54.2	34.8	7.3	0	3.7	100

received at the laboratory the fats were melted, transferred to glass jars, sealed, and stored at 4°C.

The formulas for the diets fed to the rats are shown in table 1. The casein, cod-liver oil concentrate, and salt mixture were prepared as previously described ('40). Yeast vitamins was a commercial product prepared from brewers' yeast. The label indicated that this product contained 240 B_1 and 60 B_2 Sherman units per gram. U.S.P. dextrose was used. Each diet was made up in the quantity of 1000 gm. at one

time and was stored in covered glass jars at 6°C. Diet no. 1 was practically free from fat and was fed to rats in order to determine the excretion of metabolic fat. In the diets containing 5, 15, 30, and 54.2% of fat, respectively, fat furnished approximately 12.5, 32.7, 54.5, and 77.7% of the total energy value. In the diets containing the different proportions of fat, the proportions of casein, yeast vitamins, and salts were adjusted so as to have approximately constant relationship to the total energy value.

EXPERIMENTAL PROCEDURE

The procedure in these experiments was similar to that previously followed by the writers ('40) in a study of the nutritive properties of certain animal and vegetable fats. Since the methods are described in detail in that publication, only a brief description will be given here.

The melting point of the fats was determined by the capillary tube method, the iodine number by the Hanus method, and the thiocyanogen number by the method of Kaufmann ('26). An approximately 0.1 N solution of thiocyanogen in redistilled glacial acetic acid was used. An excess of 100 to 150% of the freshly prepared reagent was used and absorption was conducted for 24 hours in the dark at room temperature (about 25°C.). Analyses were made in duplicate.

The percentages of linoleic, oleic, saturated, and unsaturated fatty acids were calculated from the iodine and thiocyanogen numbers by the formula given by Kass, Lundberg and Burr (p. 53, '40). In using this formula, the presence in lard of such a small proportion of arachidonic acid as has been reported by Ellis and Isbell ('26), and Brown and Deck ('30), would only slightly affect the calculated percentage of linoleic acid.

Before finally deciding upon the use of the formula proposed by Kass et al. ('40) in preference to that given by Jamieson ('32), some comparisons were made which showed a difference of approximately 0.4 in actual values for linoleic acid in the samples used in the present study. It is worthy

of mention that Kass et al. ('40) found elaidic acid to have the same thiocyanogen value as normal oleic acid.

In the growth experiments, all of which were conducted at the same time, each diet containing 5, 15, 30, or 54.2% of fat was fed to eight male albino rats for 60 days. The rats weighed approximately 40 gm. each at the beginning of the test and animals from different litters were distributed evenly among the different groups. Each rat was kept in an individual cage and an accurate record of food consumption obtained.

A digestion test was conducted for 7 days with each rat after the growth experiment had been in progress about 50 days. The feed consumed during the 7-day period was weighed and all feces excreted during the same period were collected. The feces were dried to constant weight at 100°C. and were analyzed for fat by the method previously described by the writers ('40). In these experiments, digestion and saponification of the feces were carried on in a 200 cc. Erlenmeyer flask with the addition of 25 cc. of 30% potassium hydroxide solution and 50 cc. of 95% alcohol.

At the end of the growth experiments, each rat was fed a fat-free diet and, after a preliminary period of at least 2 days, the feces were collected for 7 days. The feces were dried to constant weight at 100°C. and were analyzed for fat by the method previously described. The percentage of fat in the feces of a rat while on the fat-free diet was considered to be metabolic fat, and this figure was used in correcting for the metabolic fat in the feces of the same rat in the previous digestion test.

The true digestibility of each fat was calculated in the usual manner after making correction for the quantity of metabolic fat excreted during the digestion test. It was assumed that each rat excreted the same quantity of metabolic fat, in proportion to nonfatty dry matter, as when fed the fat-free diet.

STATISTICAL PROCEDURE[1]

In the analysis of the data, variance and covariance analyses as described by Snedecor ('37) were used. Adjustment of the gains to 3000 cal. was made using the within group regression coefficient between calories consumed and gain in weight. The adjustment was made to 3000 cal. merely for ease of calculation, yet this figure is within the range of the population. The minimum significant differences were obtained by multiplying the standard deviation of the mean differences calculated from the within group mean squares by the "t" values for odds of 15-1 and 99-1.

TABLE 2

Composition of fats used in feeding tests with rats

TYPE OF SHORTENING	MELTING POINT	IODINE NUMBER	THIOCYANOGEN NUMBER	FATTY ACIDS AS PERCENTAGES OF TOTAL FATTY ACIDS[1]			
				Saturated fatty acids	Unsaturated fatty acids	Oleic acid[2]	Linoleic acid[3]
	°C.			%	%	%	%
Steam-rendered lard	43	66.6	54.8	39.5	60.5	47.1	13.4
Hydrogenated cottonseed oil	39	70.8	59.2	34.5	65.5	52.3	13.2

[1] Calculated by formula given by Kass, Lundberg and Burr (pp. 53, '40).

[2] Includes isoöleic acid.

[3] May include arachidonic acid.

EXPERIMENTAL RESULTS

The composition of the fats used in the experiments is shown in table 2. It will be seen that the lard had a higher melting point and contained a higher percentage of saturated fatty acids than the hydrogenated cottonseed oil. The two products contained approximately the same proportion of linoleic acid. This hydrogenated cottonseed oil contained a considerably higher proportion of saturated fatty acids than was previously found by the writers ('40) in another brand of hydrogenated cottonseed oil. This was undoubtedly due,

[1] Acknowledgment is made to Bradford Knapp, Jr., of the Animal Husbandry Division of the Bureau of Animal Industry, United States Department of Agriculture for the statistical analysis of the experimental data.

in part at least, to the addition of fully hydrogenated cottonseed oil to the partially hydrogenated product used in the present experiments.

GROWTH-PROMOTING VALUES OF FATS

Only the average growth-promoting values of steam-rendered lard and hydrogenated cottonseed oil are shown in table 3, but the data for individual rats have been analyzed by statistical methods and pertinent results are included in the

TABLE 3

Average growth-promoting properties of steam-rendered lard and hydrogenated cottonseed oil in tests with male albino rats

TYPE OF SHORTENING	FAT IN DIET	FEED CONSUMED	GAIN IN WEIGHT IN 60 DAYS		
			Unadjusted	Adjusted to 3000 cal. intake	Per 100 cal.
	%	*Cal.*	*gm.*	*gm.*	*gm.*
Steam-rendered lard	5	3151	243	229	7.68
Hydrogenated cottonseed oil	5	2941	221	226	7.47
Difference		210	21	3	0.21
Steam-rendered lard	15	3149	267	253	8.48
Hydrogenated cottonseed oil	15	3279	260	234	7.92
Difference		130	7	19	0.56
Steam-rendered lard	30	3638	311	252	8.53
Hydrogenated cottonseed oil	30	3411	268	230	7.84
Difference		227	43	22	0.69
Steam-rendered lard	54.2	3398	297	260	8.73
Hydrogenated cottonseed oil	54.2	3193	251	233	7.86
Difference		205	46	27	0.87
Minimum significant difference (odds 19–1)		296	29	10	0.35
Minimum significant difference (odds 99–1)		394	39	14	0.46
Steam-rendered lard, average all levels		3234	280	249	8.36
Hydrogenated cottonseed oil, average all levels		3206	250	231	7.77
Difference		28	30	18	0.59
Minimum significant difference (odds 19–1)		148	15	5	0.17
Minimum significant difference (odds 99–1)		197	19	7	0.23

table. Each of the sixty-four rats used in these tests was in excellent condition at the end of the 60-day experiment, indicating that each fat was perfectly wholesome even when it constituted 54.2% by weight or 77.7% of the total energy value of the diet.

Feed consumption, expressed in terms of calories, did not differ significantly at any level of fat intake between rats fed lard and those fed hydrogenated cottonseed oil. The average feed consumption for all the rats fed diets containing lard differed only slightly from the feed consumption of all the rats fed diets containing hydrogenated cottonseed oil.

A comparison between the feed consumption of rats fed diets containing different proportions of each fat shows that in each case the feed intake was greatest when the diet contained 30% of fat. Feed consumption was least with cottonseed oil when the diet contained 5% of fat, and with lard feed consumption was lowest when the diets contained 5 and 15% of fat. The differences in feed consumption were highly significant for each fat.

The average unadjusted gains made by the rats show that, with each fat, the greatest gain was made when the diet contained 30%, and the smallest gain when the diet contained 5% of fat. The average unadjusted gain for all rats fed lard was significantly greater than the average gain for all rats fed hydrogenated cottonseed oil.

When the feed intake of all the rats was adjusted to 3000 cal., the adjusted gains show that lard and hydrogenated cottonseed oil had approximately the same growth-promoting value when the diets contained 5% of fat, but lard was definitely superior when the diets contained 15, 30, and 54.2% of fat. When the results were compared on the basis of gain in weight per 100 cal. consumed, the relative growth-promoting values of the two fats were as indicated above.

DIGESTIBILITY OF FATS

The average results of the digestion experiments with steam-rendered lard and with hydrogenated cottonseed oil

are shown in table 4. The data for each fat at each level of intake represent the average of the results obtained with eight rats. A statistical analysis of the data for individual rats shows the minimum difference necessary to be significant.

TABLE 4

Average digestibility of steam-rendered lard and hydrogenated cottonseed oil in 7-day tests with male albino rats

TYPE OF SHORTENING	FAT IN DIET	CRUDE FAT IN FECES		True digestibility
		On fat diet	On fat-free diet	
	%	%	%	%
Steam-rendered lard	5	13.61	6.48	96.18
Hydrogenated cottonseed oil	5	21.17	6.47	92.30
Difference		7.56	0.01	3.88
Steam-rendered lard	15	27.52	7.03	95.89
Hydrogenated cottonseed oil	15	42.21	7.13	90.97
Difference		14.69	0.10	4.92
Steam-rendered lard	30	36.06	6.69	95.90
Hydrogenated cottonseed oil	30	48.63	6.86	93.17
Difference		12.57	0.17	2.73
Steam-rendered lard	54.2	34.47	6.62	97.44
Hydrogenated cottonseed oil	54.2	50.49	6.62	94.96
Difference		16.02	0.00	2.48
Minimum significant difference (odds 19–1)				1.50
Minimum significant difference (odds 99–1)				1.99
Steam-rendered lard, average all levels		27.92	6.71	96.35
Hydrogenated cottonseed oil, average all levels		40.63	6.77	92.85
Difference		12.71	0.06	3.50
Minimum significant difference (odds 19–1)				0.75
Minimum significant difference (odds 99–1)				1.00

The data show that steam-rendered lard was superior in digestibility to hydrogenated cottonseed oil at each level of intake, the difference being greatest when the diets contained 15%, and least when they contained 54.2% of fat. The average

digestive coefficient for lard at all levels of intake was materially greater than the average value for hydrogenated cottonseed oil. In each case the difference was highly significant.

The digestibility of lard was practically the same regardless of the proportion of fat in the diet. On the other hand, the digestive coefficient of hydrogenated cottonseed oil was significantly higher when the diet contained 54% than when it contained 5 or 15% of fat.

It is noteworthy that, at each level of fat intake, the feces from the rats fed hydrogenated cottonseed oil contained a considerably higher proportion of crude fat than the feces from the rats fed lard. On the other hand, after the rats had been changed to the fat-free diet, there was a marked uniformity in the proportion of crude fat in the feces.

DISCUSSION OF RESULTS

The results of the present experiments concerning the relative nutritive properties of steam-rendered lard and hydrogenated cottonseed oil confirm the results of previous experiments by the writers ('40). In each series of experiments the two fats had practically the same growth-promoting value at the 5% level of intake, but lard was superior at the higher levels. Lard was superior in digestibility at all levels of intake.

In the present experiments, the available data do not supply an adequate explanation for the differences found between the nutritive properties of the two fats. As regards the relative digestibility of the two fats, although lard had a slightly higher melting point and contained a considerably higher percentage of saturated fatty acids than hydrogenated cottonseed oil, lard had the higher digestive coefficients. The percentage of stearic acid was not determined, but on the basis of available data concerning the composition of lard and cottonseed oil (Dean, '38; Hilditch, '40), it seems probable

that the two fats contained approximately the same proportion of this fatty acid. The hydrogenated cottonseed oil, of course, contained a considerable proportion of isoöleic acid. Whatever the real explanation for the differences in the digestibility of steam-rendered lard and hydrogenated cottonseed oil may be, it appears clear that the changes induced in refined cottonseed oil in the process of hydrogenation lowered its digestibility. In previous experiments ('40) the writers found that refined cottonseed oil had as high a digestive coefficient as refined steam lard, whereas hydrogenated cottonseed oil had a considerably lower value.

An explanation for the superior growth-promoting properties of steam-rendered lard as compared with hydrogenated cottonseed oil is not apparent. The two fats contained approximately the same proportions of linoleic acid, so this fatty acid apparently was not a factor. Although the percentage of linoleic acid found in the hydrogenated cottonseed oil used in this experiment may seem rather high, yet it is similar to the results obtained by the writers with a number of other samples obtained from different sources. Spadola and Ellis ('36) found 12% of linoleic acid in a partially-hydrogenated cottonseed oil with an iodine number of 68.8. The possibility has been suggested that the percentage of linoleic acid in a partially-hydrogenated cottonseed oil, as calculated from the iodine and thiocyanogen numbers, might not be valid. The reason given was that the isoöleic acids in the hydrogenated fat might have different thiocyanogen numbers than normal oleic acid. This assumption seems unwarranted since Kass, Lundberg and Burr ('40) found elaidic acid to have practically the same thiocyanogen number as oleic acid.

Although arachidonic acid was not determined in the lard used in the present experiments, it seems unlikely that such a small proportion of this acid as has been previously reported in commercial lard would materially affect the growth-promoting value of a lard such as was used by the writers.

It is noteworthy that the rat can utilize a diet containing 54.2% fat and no carbohydrates as efficiently for growth as diets containing smaller proportions of fat together with dextrose. The rats on the high-fat diets were in excellent condition throughout the experiment.

The high digestibility of steam-rendered lard at all levels of intake indicates the unusual capacity of the rat for the absorption of this fat. Although the digestibility of hydrogenated cottonseed oil was significantly lower than that of lard at each level of intake, yet the vegetable fat was well utilized even when the diet contained the maximum proportion of fat.

SUMMARY

The relative growth-promoting properties and digestibility of steam-rendered lard and hydrogenated cottonseed oil were determined by experiments with young male albino rats. Each fat was incorporated in an otherwise adequate diet in the proportions of 5, 15, 30, and 54%.

The two fats had approximately the same growth-promoting value when the diets contained 5% of fat, but lard was superior when the diets contained the larger proportions of fat. The average growth-promoting value of all diets containing lard was superior to the average value of all diets containing hydrogenated cottonseed oil.

Both lard and hydrogenated cottonseed oil induced maximum growth when the diets contained 30%, and minimum growth when the diets contained 5% of fat. Each fat was utilized least efficiently for growth when the diet contained 5% of fat, but each was utilized with approximately equal efficiency whether the diet contained 15, 30, or 54% of fat.

Lard was superior in digestibility to hydrogenated cottonseed oil at each level of fat intake. The digestive coefficients for lard ranged from 95.9 to 97.4% with an average of 96.4% whereas those for hydrogenated cottonseed oil ranged from 91.0 to 95% with an average of 92.9%.

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III. AVIAN THIAMINE DEFICIENCY

CHARACTERISTIC SYMPTOMS AND THEIR PATHOGENESIS [1]

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ONE FIGURE

(Received for publication December 17, 1940)

Thiamine deficient pigeons usually exhibit opisthotonus (head retraction) and, in fewer instances, leg weakness and hydropericardium (McCarrison, '19; Findlay, '21). The latter two symptoms have received little attention in recent years, and some investigators (Reader, '30; Kline et al., '36) have considered leg weakness (in the rat and chick) to be due to another deficiency, namely vitamin B_1. By others (Prickett et al., '39), however, it has been described in the rat as a manifestation of chronic vitamin B_1 deficiency. In humans, leg weakness and cardiac failure usually develop as a result of vitamin B_1 deficiency, whereas opisthotonus must occur rarely if at all. Spasticity (which may be related to opisthotonus) has been observed in infants.

This apparent lack of uniformity within a clinical entity is not unusual and would appear superficially to be explained adequately by individual and species differences. However, most experimental thiamine deficient diets are practically free from this factor which is a degree of restriction of thiamine in the diet rarely if ever attained in a human's ration. Moreover, various other factors, i.e., starvation, alter the severity

[1] This investigation was aided by a grant from the William W. Wellington Memorial Research Fund of Harvard University.

and course of a developing deficient state, and might possibly affect the individual variations observed so commonly in the pigeon, and be an important factor in determining the symptomatology in human beriberi.

In the present study the thiamine and food intake were varied to produce a deficiency and starvation of varying severity, and the symptomatology studied in an attempt to find a clue to the pathogenesis of the different manifestations of thiamine deficiency in the pigeon.

MATERIALS AND METHODS

Unless otherwise stated all of the observations reported in this paper were made upon white Karneaux pigeons that were 6 to 8 weeks old at the beginning of experimentation. They were fed one of three vitamin B_1 free diets. Diet I was consumed ad libitum and consisted of mixed grain (Canadian peas, Kaffir corn, yellow corn, and hard wheat) which had been autoclaved for 6 hours at 20 pounds of pressure. The behavior and food intake of birds that consumed this diet were so variable that synthetic diets II and III, which could be fed by tube, were devised. Diet II contained casein (alcohol-extracted) 20%, corn starch 65%, cod liver oil 3%, peanut oil 8%, salt mixture 4%, and vitamin K concentrate.[2] This diet was practically free of both vitamin B_1 (thiamine) and the known water-soluble members of the B complex group of vitamins. Thus in thiamine curative experiments any rehabilitation which occurred could be attributed to none other of the B group of vitamins than thiamine. Diet III contained the ingredients of diet II plus 15% autoclaved yeast, and was utilized as a control for diet II. The proportions of casein, starch, and peanut oil in diet III were changed so that the protein, carbohydrate, and fat contents of this diet were the same as in diet II.

Diet I was consumed voluntarily, but diets II and III were administered by tube as follows. The birds were secured in a

[2] Abbott.

holder;[3] a soft rubber tube ¼ inch diameter × 5 inch length was inserted into the crop; and a weighed amount of diet, previously made into a paste by the addition of an equal amount of water, was forced through the tube from a catheter syringe. This method of feeding diets II and III was employed with no harmful results in more than 300 pigeons, and some birds were fed diet III in this manner for as long as 6 and 8 months. Thiamine supplements[4] were dissolved in distilled water and injected intramuscularly.

Subsequent to preliminary experimentation with both diets II and III, the food (diet II) and thiamine intake were varied in sixty pigeons (fig. 1). Twenty-four of these were each fed 20 gm., and the remaining thirty-six either 10 or 5 gm. of diet II daily. After two birds in each group vomited, all birds in that group were given a daily supplement of thiamine intramuscularly, ranging from 0 to 25 μg. for birds on the 20-gm. intake, 0 to 12 μg. for those on 10-gm. intake, 0 to 7 μg. for those on the 5-gm. intake. These birds were carefully observed daily for signs of opisthotonus, ataxia, leg weakness, decreased activity, regurgitation and dyspnea, and when these symptoms had been present for a desired period of time, 50 to 200 μg. of thiamine were given intramuscularly each day until partial or complete rehabilitation had occurred. During the period of repair most birds continued to receive diet II, but some of them, for the sake of convenience, were fed normal grain.

Many other pigeons were used for studies relative to the production of hydropericardium and other findings compatible

[3] The holder is made of ¾ inch wood stock on a base 12 inches × 6 inches. Four small pieces of wood are mounted edgewise and at angles to fit the contour of the pigeon's body when on its back with wings closed. One side of two pieces at 150 degree angle is fixed to the base. The other two pieces can be moved in slots and about the axis of bolts, used to secure their position, in a manner to obtain a snug fit. One slot is parallel and the other at a 45 degree angle to the edge of the base. An oblong enclosure of varying dimensions is thus obtained. The pigeon's head protrudes through a hole formed by matched notches in the two end pieces when they are in position.

[4] Betabione, Merck.

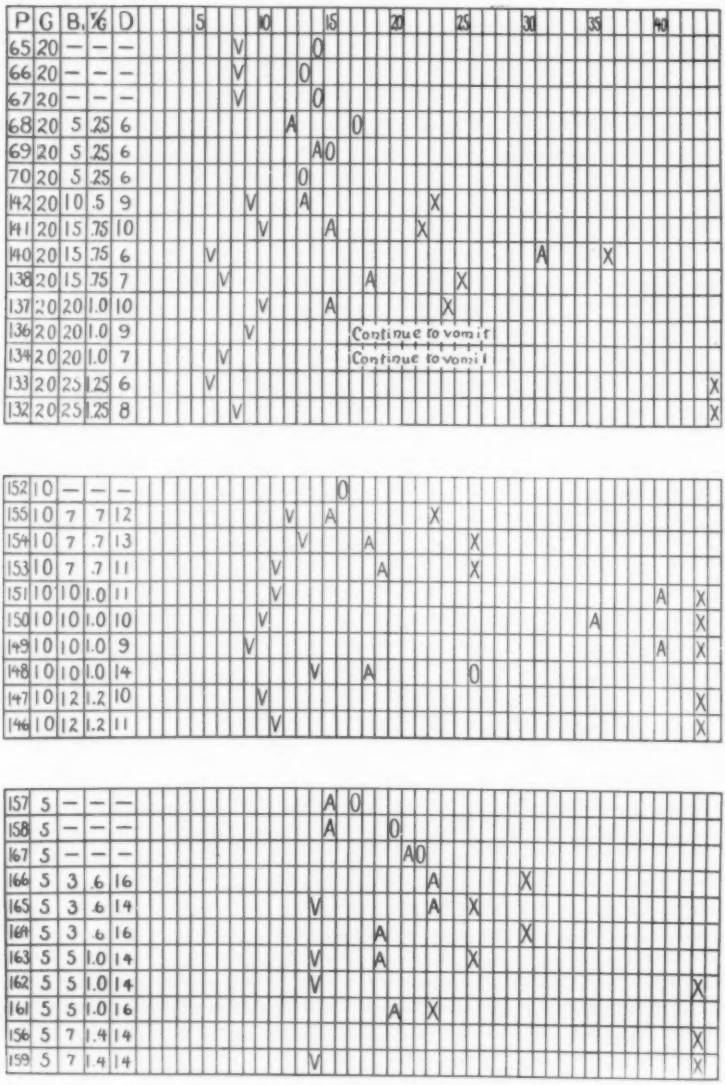
P G B. % D
5 10 15 20 25 30 35 40
65 20 — — —
66 20 — — —
67 20 — — —
68 20 5 .25 6
69 20 5 .25 6
70 20 5 .25 6
142 20 10 .5 9
141 20 15 .75 10
140 20 15 .75 6
138 20 15 .75 7
137 20 20 1.0 10
136 20 20 1.0 9
134 20 20 1.0 7
133 20 25 1.25 6
132 20 25 1.25 8
Continue to vomit
Continue to vomit
152 10 — — —
155 10 7 7 12
154 10 7 .7 13
153 10 7 .7 11
151 10 10 1.0 11
150 10 10 1.0 10
149 10 10 1.0 9
148 10 10 1.0 14
147 10 12 1.2 10
146 10 12 1.2 11
157 5 — — —
158 5 — — —
167 5 — — —
166 5 3 .6 16
165 5 3 .6 14
164 5 3 .6 16
163 5 5 1.0 14
162 5 5 1.0 14
161 5 5 1.0 16
156 5 7 1.4 14
159 5 7 1.4 14

Figure 1

with cardiac failure. The details of these experiments will be described in the text.

RESULTS

Diet I. The behavior of thirty or more birds that consumed diet I (heated mixed grain) voluntarily was essentially the same as was noted by McCarrison ('19) and Findlay ('21). Ataxia appeared on about the nineteenth day and was followed in 1 to 13 days by opisthotonus. Anorexia was usually noticed by the seventh to tenth day, and nearly every bird lost 20 to 35% of its original weight by the time that neurological symptoms had appeared. One pigeon that was given inadequate thiamine supplements for 11 weeks and then deprived of thiamine entirely developed cardiac failure with pulmonary edema and hydropericardium.

Diet II (fed by tube). It was known from preliminary experimentation that a pigeon could maintain or gain weight and remain active and in good health for 8 to 12 weeks, when fed by tube a daily ration of 20 gm. of diet II plus an intramuscular injection of 25 μg. or more of thiamine. Later, anorexia, a loss of weight and anemia appeared, and were not influenced by increasing the thiamine intake. Consequently no experiments with diet II exceeded 8 weeks, and, unless specifically stated, none exceeded 6 weeks in duration.

Reference to figure 1 shows that vomiting [5] (anorexia) occurred on or about the eighth experimental day in almost all birds receiving 20 gm. of diet daily and no thiamine. This

[5] The food was spilled apparently by lowering and shaking the head.

Fig. 1 The clinical observations upon birds that were fed 20, 10, and 5 gm. of diet II daily are included in the figure. In the first vertical column (P) is to be found the number of all pigeons referred to by number in the paper; in the second column (G) is noted the daily ration (in grams of diet II); in the third (B_1) can be found the daily thiamine intake (in micrograms); in the fourth (γ/g) the ratio of micrograms of thiamine/grams of diet II; and in the fifth (d) the day on which thiamine was started. The horizontal row of numbers from 5 to 40 at the top of the chart gives the experimental day. The letters in the chart have the following meaning: V—Vomiting; O—Opisthotonus (immediately repaired or sacrificed); A—Ataxia; X—Experiment discontinued; pigeon given reparative doses of thiamine or sacrificed.

was both delayed and reduced in quantity by reducing the daily ration to 10 gm., and absent or greatly delayed in birds that received 5 gm. of diet II daily.

When thiamine was added the vomiting was decreased roughly proportional to the size of the dose so that many of the chronically deficient pigeons lost no food by this means.

Due to this plan of feeding and the behavior of the birds under these conditions, the average loss of weight at the time neurological signs first appeared was only 5% in those receiving 20 gm.; 15% in those receiving 10 gm. and 24% in those receiving 5 gm. of food, in marked contrast to the large uncontrollable losses in weight which usually occur with voluntary feeding.

Neurological manifestations

The clinical manifestations of thiamine deficiency in these experiments varied (fig. 1) depending upon the rate of onset of the deficiency and this rate was dependent upon the ratio of thiamine intake to food intake. The acute deficiency produced by feeding the thiamine-free diet in amounts sufficient to prevent a marked weight loss always led to opisthotonus except in very old birds (P. 65, 66, 67).[6] The more acutely the deficiency developed, the greater the tendency seemed to be for opisthotonus to be severe and sustained, accompanied by backward rolling and extensor rigidity of the legs, and flapping of the wings. When the deficiency developed less rapidly because of concomitant starvation (157, 158 and 167) or the addition of small amounts of thiamine to the diet (68, 69, and 148) ataxia preceded the opisthotonus. If the thiamine supplements were greater and the deficiency in thiamine developed slowly, ataxia and later leg weakness appeared (137, 138, 140, 141, and 142).

The earliest evidence of leg involvement was ataxia, which appeared as early as the thirteenth day, but usually later.

[6] All pigeons referred to by number are to be found in the chart.

It was characterized by a wide gait, misplacing of the feet, anterior buckling of the knees, and staggering and was frequently followed by definite muscular weakness so that the birds were unable to support their weight. The paralysis developed rapidly when the thiamine intake was low, and in 1 to 3 days the birds were unable to stand, although they could still flap their wings and support their heads with no obvious difficulty. Many birds were maintained in this state for 7 to 14 days, and one for 4 weeks, after which they remained paralyzed for a variable period during repair. A few birds with a nearly adequate thiamine intake exhibited definite mild ataxia 1 day and normal gait the next, for as long as 3 weeks before the paralysis became definitely sustained, and marked leg weakness, without buckling of the knees and misplacing of the feet, could be produced temporarily in others by exercise.

All cases of leg weakness or opisthotonus that appeared in animals on diets I, II, and III were alleviated by therapeutically adequate intramuscular injections of thiamine. Opisthotonus of short duration was relieved in approximately 1 or 2 hours by 50 to 100 μg. of thiamine, but smaller doses of thiamine required as long as 8 to 12 hours. A few birds on the starvation and deficient diets (and a few receiving autoclaved grain) recovered spontaneously from opisthotonus for a day or so, but in none of the well-fed acutely deficient birds was this observed. This was thought to be due to the ability of these animals to give up their tissue stores of thiamine, or to various other factors which conserve the animal's supply of vitamin B_1, i.e., fasting, vomiting, and coprophagy. The symptoms of ataxia or leg weakness were abolished much more slowly. Birds with only ataxia for a day or less were entirely free of symptoms in 1 or 2 days on a dosage of 50 to 100 μg. of thiamine, but leg weakness (paralysis) severe enough to make standing difficult for 3 days, delayed complete clinical rehabilitation for 2 to 4 weeks (three experiments), and if the paralysis had existed for 7 to 9 days, restoration to normal was delayed for 6 to 8 weeks (six

experiments). A number of birds with moderate leg weakness improved spontaneously for a few days while being maintained on thiamine intake slightly inadequate for their daily need, although no change in the food or thiamine intake was made. Later the paralysis reappeared and was abolished only after definite curative doses of thiamine were given. Although barely adequate doses of thiamine were curative, it seemed wise in these experiments to give what was found later to be a large excess (50 to 100 μg. daily).

Recovery from leg weakness was successful in nine birds that received diet II plus 50 to 100 μg. of thiamine daily. Two of these with severe symptoms improved slowly while receiving the starvation ration of 5 gm. daily, but much more rapidly after the food intake was doubled, although the thiamine intake was unchanged and the caloric intake still insufficient for normal growth. Two other birds that were recovering satisfactorily on diet II plus thiamine, developed other deficiencies after the experiment had exceeded the 8 to 12 week limit and exhibited anemia and weight loss, but an increase in the paralysis was not noted.

The data in figure 1 confirm the previously reported (Cowgill, '39) dependence of thiamine requirements on food intake; 1 gm. of diet II requires about 1.25 μg. of thiamine to prevent all symptoms. Thus it is not surprising that birds which continue to vomit (134, 136) failed to develop paralysis; other birds (140, 138, 151, 150, 149) that vomited early, developed ataxia late; and those that vomited late (141, 148) developed ataxia early. The vitamin economy affected by vomiting is also illustrated by older birds that received 20 gm. of diet II daily and failed to vomit. They occasionally developed opisthotonus in 9 to 11 days, whereas the younger birds that vomited required 13 to 15 days and occasionally longer to develop this symptom.

Concomitant starvation tends to make a deficiency state appear more chronic (157, 158, 167) probably because a lowered food intake allows the rapidly depleting tissue stores

to be used at a slower rate, so that these birds resemble those on higher food intake which receive small supplements of thiamine (68, 69, 148).

Cardiac failure: its production and alleviation

Evidences of cardiac failure (pericardial effusion, dyspnea, engorgement and edema of lungs, engorgement of the liver and (or) dependent edema of the body) were observed rarely in the acutely deficient pigeons (such as 65, 66, 67) with opisthotonus only, and never in the severely starved and deficient ones. However, in a group of eleven chronically deficient birds with leg weakness that received a daily ration of approximately 15 gm. of food and 5 to 10 μg. of thiamine for 25 to 32 days (similar to 68, 69, and 70), nine had one or more postmortem evidences of incidental cardiac failure, and two that had been given large doses of thiamine for purposes of repair were found normal at autopsy 4 days and 1 month later. Of those cases with evidence of cardiac failure, pericardial effusion, apparently alone, was found in three cases, with pulmonary edema in four, and pulmonary engorgement of the liver and edema of the pectoral regions and thighs in one. In no one of these eleven chronically deficient pigeons was dyspnea or other antemortem evidence of cardiac failure observed.

In ten other chronic cases on a similar chronic dietary regimen, severe acute cardiac failure indicated clinically by dyspnea was produced by suddenly discontinuing the thiamine. These birds had received a daily ration of 20 gm. of diet II, and, subsequent to vomiting, 10 to 20 μg. of thiamine for 1 to 4 weeks. The thiamine was then withdrawn, and 1 to 4 days later dyspnea developed. Despite thiamine medication five of these birds died less than 1 hour after dyspnea was noted and their postmortem studies revealed very marked engorgement and edema of the lungs, engorgement of the liver and pericardial effusion. Occasionally this severe type of cardiac failure with dyspnea was seen in birds that were maintained on a prolonged chronic deficiency regime with a

continued intake of inadequate doses of thiamine, and a few of these birds developed opisthotonus terminally. It should be noted that some pigeons with either incidental or severe cardiac failure had slight to moderate leg weakness, but opisthotonus was observed rarely except as a terminal event.

Incidental cardiac failure was readily eliminated by injections of thiamine as was indicated by the absence of these pathological findings in all the successfully treated birds included in the chart. Furthermore, in a group of twelve chronically deficient birds, all of which were treated in an identical fashion, hydropericardium and other evidences of incidental cardiac failure were observed at postmortem in six birds, and one appeared normal. The five remaining birds were given 100 μg. of thiamine daily and autopsied after 1, 2, 3, 4, and 5 days. No evidence of cardiac failure could be found. On the other hand, five pigeons with severe cardiac failure and dyspnea failed to respond to thiamine and died within a few minutes of its administration. Five others, however, responded to this medication and were apparently normal in 30 to 60 minutes.

Diet III (fed by tube). The foregoing experimentations with diet II were repeated with diet III on fewer birds. The clinical observations were essentially the same in the two groups, so this group will not be described separately.

DISCUSSION

It appears that pigeons allowed to feed voluntarily on thiamine deficient diets will unpredictably become acutely or chronically deficient, depending upon the intensity of their appetites and the amount of thiamine which they consume. A few birds will eat the deficient diet voraciously and develop opisthotonus in 14 days while others, because of starvation, will develop a chronic deficient state or die from anorexia. This is the principal reason for the general failure to consistently produce satisfactory opisthotonus in pigeons for assay purposes.

If the diet consists of poorly-milled rice or mixed grain insufficiently autoclaved, or if wire-bottomed cages are not used or are cleaned poorly so that fecal matter is available, an unknown and occasionally adequate amount of thiamine will be obtained. Under such circumstances, pigeons will develop a chronic deficiency (no opisthotonus) or none at all.

It is important to recognize that acute and chronic thiamine deficiency, although different in many respects, represent two phases of the same deficiency. It seems probable that many of the inconsistencies in the literature concerning vitamin B_1 deficiency are due to a failure to recognize that symptoms of a deficiency may vary depending upon the manner in which the deficiency is produced. This ought to be kept in mind, especially when proposing a new factor as an explanation for inconsistent symptoms and lesions. The vitamin B_4 hypothesis seems to be a case illustrating this point.

The immediate response of the acutely deficient birds with opisthotonus to thiamine treatment indicates that faulty function is primarily responsible for this symptom. The alleviation of mild ataxia and some prompt but limited improvement in leg weakness indicate that a functional factor is also probably involved in the chronic deficiency. However, the fact that complete rehabilitation of leg weakness requires from several days to several weeks, depending upon the time at which repair is instituted, is almost conclusive evidence that anatomical changes are also involved in these cases. These observations have a bearing on the controversial point as to whether peripheral nerve degeneration is involved in thiamine deficiency. The above evidence plus the detailed pathological findings reported elsewhere (Swank, '40) show that leg weakness is accompanied by peripheral nerve degeneration, the extent of which depends on the time factor; and that upon repair with thiamine on a diet entirely devoid of other factors of the B complex, the peripheral nerves regenerate parallel with clinical improvement in the symptoms. It seems clear that experimental thiamine deficiency can exist with symp-

toms due either to a functional or an anatomical lesion, or both, depending upon the manner in which it is produced.

One cannot dismiss the possibilities that species differences may also influence the type of symptoms which will develop. Perhaps opisthotonus is produced easily in the pigeon because of the highly developed vestibular system. In ducks, opisthotonus was also produced with ease, but in chickens with greater difficulty, although identical dietary methods were used. The age is also an important factor; leg weakness and opisthotonus were most easily produced in young growing or adult birds, while old birds have a marked tendency to develop less conspicuous symptoms.

These experiments suggest that man usually develops the chronic type of thiamine deficiency (muscular tenderness, leg weakness and cardiac failure or both). This is consistent with the fact that his diet is usually not entirely free of thiamine. Dogs also develop the chronic type of thiamine deficiency as they conserve their thiamine by voluntary starvation (or vomiting when tube-fed). In a separate study of thiamine deficiency in dogs (Swank et al., '41) in which tube-feeding was used, one dog that did not regurgitate developed extensor rigidity with opisthotonus. This was abolished within a short time by an intramuscular injection of thiamine. This observation and the fact that spasticity occurs in infants with beriberi suggest that opisthotonus might develop in man if a diet entirely free of thiamine were consumed in an amount sufficient to prevent a significant loss of weight.

In spite of possible species differences it would seem worthwhile to consider some of the observations reported here as contributing toward a better understanding of the human deficiency.

SUMMARY AND CONCLUSIONS

Young pigeons fed a highly purified thiamine-free diet by tube in quantities sufficient to prevent large weight loss invariably developed an acute thiamine deficiency characterized by opisthotonus. When the ration was made only partially

adequate by additions of thiamine, ataxia and leg weakness developed in all pigeons and cardiac failure in many.

The intramuscular administration of thiamine promptly relieved the opisthotonus and the mild cardiac failure. Leg weakness, however, was restored to normal only slowly, irrespective of the presence or absence of other factors of the "B complex" and severe cardiac failure often did not respond to treatment.

The thiamine requirement varied with the food intake, 1.25 μg. per gram of diet II were required for pigeons to remain symptom-free.

The assumption that the absence of a hypothetical factor (vitamin B_4) is responsible for the development of a paralysis when the thiamine intake is low is unnecessary in the case of pigeons. This paralysis is a characteristic symptom of chronic thiamine deficiency. The question is raised as to whether this is not also the case for the chick and rat.

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THE EFFECT OF DIETARY CALCIUM AND PHOSPHORUS ON THE ASSIMILATION OF DIETARY FLUORINE [1, 2]

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(Received for publication January 3, 1941)

There is a definite fluorine hazard in the United States and throughout the world. The occurrence of fluorine in drinking water is general and in many communities, especially those served by deep wells, the concentration is such (0.9 p.p.m. or more) as to cause mottled enamel in children during the period of tooth formation (Nichols, '39). McClure ('39) has summarized published analyses of the distribution of fluorine in foods. Excluding excessive values obtained in certain fluorite areas and values obtained for vegetables and fruits sprayed with fluorine-containing insecticides, some 42% of the analyses range from 0 to 0.9 p.p.m. on the dry basis, 35% range from 1 to 2.8 p.p.m. (the present Federal tolerance for fluorine for marketable sprayed produce), and 23% range above 2.8 p.p.m. In the latter classification are sea foods, teas, baking powders and some milling by-products and leguminous seeds. According to this summary, foods grown in fluorite areas may show greatly increased contents of fluorine. The considerable use of fluorine-bearing insecticides in the fruit industry (Carter and Busbey, '39), especially

[1] This experiment was made possible by the donation of funds to the University of Illinois by the Aluminum Company of America and the Pennsylvania Salt Manufacturing Company.

[2] This investigation was conducted under the supervision of a Committee on the Physiological Effects of Spray Chemicals, appointed by the director of the Agricultural Experiment Station and consisting of the following members: H. H. Mitchell, W. A. Ruth, W. P. Flint and Julia P. Outhouse.

in the northwest, accounts for another item in the usual fluorine hazard to which the population of this country is exposed. A further item, of no general prevalence it is true, is the exposure to fluorine in industry (Machle and Evans, '40).

Some of these items in the usual fluorine hazard are beyond control, while some are subject to control within limits, depending upon economic factors, so that the fluorine hazard cannot be removed or reduced below certain limits. It is important, therefore, to consider the possibility of alleviating the physiological effects of fluorine on the body by dietary means, in order intelligently to be able to minimize the deleterious effects of fluorine consumed in food and water in amounts that it is either impossible or inexpedient to reduce.

The possibility of modifying the physiological effects of fluorine by dietary means has been explored with varying results. In an earlier paper from this laboratory (Lawrenz, Mitchell and Ruth, '39 b) it was shown that fluorine in food is retained in the body to a less extent (about 20% less) than fluorine in water, when the water is consumed at times such that admixture with food in the stomach cannot occur. The failure of McClure ('39) to confirm this finding may be ascribed to the fact that in his experiments the consumption of water was not regulated, and since dry food was offered the rats, a preponderant share of the day's water intake was consumed simultaneously with the food.

In connection with such studies, it seems fair to presume that the effect of any dietary factor upon the toxicity of fluorine reflects accurately its effect on the retention of fluorine in the body, since no mechanism is known, or seems at all probable, by which fluorine could be retained in some innocuous form. Hence, the degree of retention of fluorine under a given set of experimental conditions may be taken to measure the intensity of the physiological effects of dietary fluorine. It possesses the additional advantage of being amenable to quantitative and exact measurement.

Smith ('35–'36) has reported the results of a general study of the effects of dietary factors on fluorine toxicity as

measured by the time of appearance of tooth striations and of bleaching of the dental enamel in the rat and by the severity of these effects. Excessive supply of dietary essentials did not nullify these effects of fluorine, while suboptimal intakes of any known dietary essential, including calcium, were not observed to induce any increased susceptibility to dental fluorosis, although additive effects were evident if these dietary deficiencies in themselves were capable of impairing tooth formation. However, as De Eds ('33) has shown in his excellent review of chronic fluorine intoxication, there have been many indications that dietary calcium exerts a protective action and that calcium deficient dietaries accentuate the symptoms of fluorosis. In fact, before the etiology of mottled enamel was established, Pierle ('26) concluded that a low-calcium diet could produce mottled and stained teeth. Hauck, Steenbock and Parsons ('33) reported that the growth of rats was poorer on a food containing 0.15% NaF when the calcium content was low than when it was adequate, and that a supplement of vitamin D reduced the toxicity of the calcium-poor food, but not that of the calcium-rich. Schulz ('38) has recorded similar experiences, largely of a qualitative nature.

In a recent publication from this laboratory (Shields and Mitchell, in press) it was shown that dietary calcium and phosphorus are protective against lead retention in the sense that moderate as compared with low dietary concentrations of these elements depress greatly the retention in the body of dietary lead. Because of this relationship, which had been observed (though not very precisely defined) before the initiation of the work above referred to, and because of rather clear indications, not without some seemingly contradictory evidence however, that calcium is protective against fluorine retention, the experiments described in this report were undertaken. Their object was to define as accurately as possible any relationship that may exist between the concentrations of dietary calcium and phosphorus and the retention of dietary fluorine. An essential feature of the investigation was the control of all gross factors, including the intake of food, that would be

expected to modify the retention of fluorine. The dietary fluorine was purposely kept low so that the results could be more readily applied to the fluorine hazard existing in practical nutrition.

PLAN OF THE EXPERIMENTS

The experiments were carried out on growing albino rats. The first experiment was concerned with the effect on fluorine retention of variable concentrations of dietary calcium, the concentration of dietary phosphorus being kept constant. Twelve pairs of littermate rats of an initial average body weight of 120 gm., and eight pairs of younger littermates, averaging 44 gm. in weight were used in comparing the effects of a low-calcium and of a high-calcium diet. The low-calcium diet contained 47.5% of ground yellow corn, 46% of dried whole egg, 3% of dried yeast [3], 2% of a fortified cod liver oil [4] and 1.5% of $BaSO_4$. To this ration a synthetic cryolite was added in a concentration equivalent to 8 p.p.m. of fluorine. By actual analysis, the ration contained 0.23% of calcium and 9.4 p.p.m. of fluorine. The ratio of Ca to P was 0.44 to 1.

One rat in each of the twenty pairs of rats received this low-calcium ration, while the other rat received a high-calcium ration prepared according to the above formula except for the substitution of $CaCO_3$ for the $BaSO_4$. The high-calcium ration contained by actual analysis 0.73% of calcium and 9.4 p.p.m. of fluorine. In this case, the Ca : P ratio was 1.40 to 1.

The paired rats received the same amounts of their respective diets and were fed to the limit of their appetites. The feeding period for each pair was terminated when each rat had consumed 1 kg. of food, with the exception of two pairs in the group of larger rats. One rat in each of these pairs developed abscesses on the neck, bearing no apparent relation to the experimental treatment, and the two pairs were sacrificed after consuming only 557 and 627 gm. of food, respectively.

[3] Northwestern Yeast Co., Chicago, Ill.

[4] Nopco XX.

per rat. At the termination of the feeding experiment, all rats were sacrificed for analysis, and at the beginning of the experiment, sample rats were taken for analysis from each of the litters that furnished rats for the experiment. These two series of analyses permitted the estimation of fluorine retention during the course of experimental feeding.

In the second experiment, a similar comparison was made among three rations containing the same concentrations of calcium and fluorine, but three different levels of phosphorus. The basal (low phosphorus) ration contained 7% of egg albumin, 10% of casein, 8% of dried yeast, 10% of sucrose, 1.4% of a salt mixture free of calcium, phosphorus and fluorine, 6% of corn oil, 6% of lard, 2% of cod liver oil, 0.5% of wheat germ oil, 46.05% of starch, 3.05% of $CaSO_4.2H_2O$, and sufficient synthetic cryolite to provide 10 p.p.m. of added fluorine. The phosphorus contents of the other two rations were adjusted to the desired levels without disturbing the calcium content by substituting for the $CaSO_4.2H_2O$ appropriate mixtures of $CaHPO_4$, $CaSO_4.2H_2O$, and $BaSO_4$. The three rations contained, respectively, 0.14, 0.54 and 0.71% of phosphorus and 0.71% of calcium, but instead of the anticipated 10 to 12 p.p.m. of fluorine, they were found to contain 32 p.p.m., attributable to a brand of commercial casein with a high content of fluorine.

Four trios of rats, averaging 48 gm. in initial weight, were used in the comparison of these three rations. The experiment was terminated with the consumption of 500 gm. of food per rat, and the fluorine storages were estimated in the usual way.

The third experiment was similar to the second in purpose and plan. In order to reduce the fluorine content to the desired level, egg albumin was substituted for casein in the experimental rations, which contained, respectively, 0.18, 0.51, and 0.63% of phosphorus, 0.74% of calcium and approximately 13 p.p.m. of fluorine. Eight trios of rats were used in these comparisons, which were terminated after the consumption of 600 gm. of food per rat.

The experimental rats were weighed weekly and after the sixth week of feeding, the teeth were examined weekly for the appearance of striations, using a jeweler's lens with a magnification of 4x. At the completion of the feeding period, the rats were killed with ether and the empty weights and body lengths taken. The carcasses were then autoclaved and the flesh, bones and teeth separated and analyzed for fluorine by methods previously described (Lawrenz, Mitchell and Ruth, '39 a). In the first experiment the dried bones were also analyzed for ash, calcium and phosphorus by approved methods.

DISCUSSION OF RESULTS

The effect of low- and high-calcium levels on fluorine retention. Striations began to appear on the teeth rather generally among the low-calcium rats during the eighth week, and among the high-calcium rats either simultaneously with their pair mates or shortly thereafter. The low-calcium rats gained at a somewhat slower rate than their pair mates, but the differences were not sufficiently distinct to be significant. After periods averaging 106 and 98 days for the groups of small and large rats, respectively, the rats were killed and analyzed. The average results of the various measurements and analyses made on the carcasses are summarized in table 1, with statistical analyses of their significance according to the method of Student ('25). The larger and smaller rats are grouped separately in this table.

The high-calcium diet produced a distinctly heavier weight of dry fat-free bone, containing significantly higher percentages of ash, calcium and phosphorus. On the other hand, compared with the low-calcium diet, it depressed the total retention of fluorine by 10.5% for the smaller rats, and 12.8% for the larger rats. While quantitatively most of this depression of fluorine retention occurred in the bones, the percentage depressions were more marked in the teeth and in the soft tissues. The percentage decreases in fluorine content for the

TABLE 1

The effects of low and high levels of dietary calcium on growing rats, with particular reference to the disposition of the dietary fluorine.

	DATA FOR 8 PAIRS OF SMALLER RATS					DATA FOR 12 PAIRS OF LARGER RATS				
	Averages		Differences between pair-mates		Probability of a fortuitous outcome	Averages		Differences between pair-mates		Probability of a fortuitous outcome
	Low-Ca rats	High-Ca rats	Average	Standard deviation		Low-Ca rats	High-Ca rats	Average	Standard deviation	
Initial body weight, gm.	44	44	.....	.....	.....	120	121	.....	.....	.....
Gain in body weight, gm.	232	240	8.5	17.8	0.12	157	160	2.92	16.3	0.28
Final body length, mm.	221	224	Insignificant			225	228	Insignificant		
Days on experiment	106	106	.....	.....	.....	98	98	.....	.....	.....
Food consumed, gm.	1000	1000	.....	.....	.....	1000	1000	.....	.....	.....
F consumed, mg.	9.40	9.42	.....	.....	.....	9.40	9.42	.....	.....	.....
Dry weight of fat-free bones, gm.	8.030	12.104	Highly significant			10.176	12.075	Highly significant		
F in bones, p.p.m.	460	280	180	41.8	<0.00005	403	301	102	36	<0.00001
F in bones, mg.	3.692	3.366	0.326	0.298	0.012	4.103	3.633	0.470	0.283	0.0001
Dry weight of teeth, gm.	0.4489	0.4618	0.129	0.370	0.19	0.4651	0.4660	Insignificant		
F in teeth, p.p.m.	232	171	61.4	21.5	0.00007	206	165	41.2	27.3	0.0002
F in teeth, mg.	0.104	0.079	0.0255	0.0141	0.0010	0.096	0.077	0.0197	0.0127	0.0002
Fresh weight of tissue, gm.	260	260	Insignificant			257	257	Insignificant		
F in soft tissues, p.p.m.	0.70	0.54	0.159	0.103	0.0024	0.60	0.60	Insignificant		
F in soft tissues, mg.	0.181	0.138	0.0429	0.0377	0.0098	0.151	0.148	0.0206 [2]	0.0262	0.016
Empty carcass weight, gm.	276	284	.....	.....	.....	277	281	.....	.....	.....
F in carcass, mg.	3.976	3.583	.....	.....	.....	4.351	3.857	.....	.....	.....
F retention,[1] mg.	3.738	3.346	0.392	0.319	0.007	3.857	3.362	0.499	2.70	<0.00001
F retention, pct.	39.77	35.52	.....	.....	.....	44.63	38.98	.....	.....	.....
Ash in dry bones, pct.	42.85	49.85	7.00	3.51	0.0006	46.92	49.99	3.07	3.61	0.0086
Ca in dry bones, pct.	15.54	18.55	3.01	1.44	0.0005	17.23	18.65	1.42	1.39	0.0031
P in dry bones, pct.	8.26	9.35	1.09	0.680	0.0019	8.82	9.29	0.474	0.707	0.024
Ca: P ratio in bones	1.88	1.98	.....	.....	.....	1.95	2.01	.....	.....	.....
Ca: F ratio in bones	340	670	.....	.....	.....	430	626	.....	.....	.....
P: F ratio in bones	181	338	.....	.....	.....	220	312	.....	.....	.....

[1] The initial fluorine content of check rats was found to be 5.4 p.p.m. of live weight for the smaller rats and 4.1 p.p.m. for the larger.

[2] After eliminating one aberrant difference by means of Chauvenet's criterion.

smaller and the larger rats were, respectively, 8.8 and 11.5 for the bones, 24.5 and 20.5 for the teeth, and 23.7 and 13.6 for the soft tissues.

From these values one may infer that the protective effect of an increase in the calcium content of the diet from an inadequate (0.23%) to an adequate (0.73%) level is greater than the total depression of fluorine retention would indicate, since the most harmful effects of fluorine are presumably exerted on the teeth and on the soft, or protoplasmic, tissues.

Also the average values cited above would indicate that the protective action of calcium is greater with respect to the teeth and the soft tissues for the younger rats than for the older. However, a statistical analysis of the two sets of data failed to reveal conclusive evidence to that effect, although the evidence was quite indicative ($P = 0.075$) with reference to the soft tissues.

The effect of variable intakes of dietary phosphorus on fluorine retention. In experiments 2 and 3, the low-phosphorus diets were poorly consumed and the growth of all trios was consequently very slow. On equal intakes of food, the rats on the two higher levels of phosphorus exhibited significantly greater gains in body weight and attained significantly greater body lengths. Also, in experiment 3, the high-phosphorus rats gained in weight significantly more than the medium-phosphorus rats and attained significantly greater body lengths.

However, the results of the carcass analyses in these two experiments were largely negative in significance. Although the amounts of fluorine retained averaged higher for the low-phosphorus rats than for the medium-phosphorus rats in both experiments, the differences were statistically quite insignificant, the probability of a fortuitous outcome being 0.33 in experiment 2 and 0.28 in experiment 3. The differences between the medium- and high-phosphorus rats were also clearly insignificant. An average of 36.6% of the fluorine intake was retained in experiment 2, and 30.7% in experiment 3. The total fluorine contents of the bones, teeth and soft tissues also

were not significantly affected by the variable intake of dietary phosphorus in both experiments.

In both experiments, the average weight of dry fat-free bones was greater by about 14% in the medium-phosphorus rats than in the low-phosphorus rats and the difference was highly significant. In experiment 3, but not in experiment 2, the weight of dry fat-free skeleton increased significantly by about 7% in the high-phosphorus rats as compared with their pair-mates on the medium-phosphorus diet.

Since the total fluorine content of the skeleton was not significantly affected by the concentration of phosphorus in the diet, while the weight of dry fat-free skeleton was affected, the concentration of fluorine in the bone was modified by the variable phosphorus intake. In experiment 2, in which a higher level of fluorine was fed, the concentration of fluorine in the dry fat-free bone averaged 793, 687, and 662 p.p.m., respectively, for the low-, medium-, and high-phosphorus rats. Only the difference between the low- and the medium-phosphorus rats is significant. In experiment 3, the average concentrations in the three groups of rats were 378, 322, and 299, respectively, and both differences between successive averages are significant.

Evidently, within the range studied, variation in the concentration of phosphorus in the diet does not appreciably modify the assimilation of low levels of dietary fluorine.

SUMMARY AND CONCLUSIONS

In three experiments, involving a total of seventy-six rats, the effects of varying levels of dietary calcium, dietary phosphorus remaining constant, and of dietary phosphorus, dietary calcium remaining constant, on the retention and distribution of low levels of dietary fluorine (9, 12 and 32 p.p.m.) among skeleton, teeth and soft tissues were determined. The experiments involved equalized feeding of rats on comparable rations, analysis of check rats at the start of the experiments and analysis of all experimental rats at the termination of feed-

ing periods during which 1000 gm., 500 gm., and 600 gm. of food were consumed, respectively, per rat. The results secured warrant the following conclusions:

1. An increase in the concentration of dietary calcium from 0.23 to 0.73%, phosphorus remaining constant, produces a distinctly heavier dry fat-free skeleton, containing significantly higher percentages of ash, calcium and phosphorus in the growing rat. On the other hand, it depresses the total retention of fluorine by 10 to 13%, and to a greater extent the deposition of fluorine in teeth and soft tissue. Quite probably this protective action of calcium against assimilation of fluorine is greater in young than in older rats.

2. An increase in the concentration of dietary phosphorus from 0.14 to 0.71%, dietary calcium remaining constant, produces in young growing rats a greater appetite for food, a slightly greater rate of growth and a distinctly heavier dry fat-free skeleton. However, it does not modify appreciably the total retention of fluorine or the distribution of retained fluorine among bones, teeth and soft tissue. The increased weight of skeleton with no increase in its content of fluorine, does, however, depress the concentration of fluorine in the dry fat-free bones.

3. Presumably dietary calcium consumed in concentrations above certain minimum (inadequate ?) levels, protects the body against dietary fluorine, in food or in water, by impairing its assimilation to some extent, especially in those tissues, the teeth and the soft tissues, where its most deleterious effects would be exerted.

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